Genómica Funcional del Metabolismo del Nitrógeno en relación con la fotorrespiración y estrés abiótico en plantas de *Lotus japonicus*.

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Genómica Funcional del Metabolismo del Nitrógeno en relación con la fotorrespiración y estrés abiótico en plantas de *Lotus japonicus*.

Trabajo presentado para optar al grado de Doctora en Química por la licenciada

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A mi familia

A Juandi

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RESUMEN

En esta Tesis Doctoral se ha abordado el estudio, desde el punto de vista de la genómica funcional, del metabolismo del nitrógeno y su relación con la fotorrespiración y con el estrés abiótico en la leguminosa modelo *Lotus japonicus*. La disponibilidad de mutantes *Ljgln2-2*, carentes por completo de actividad y proteína glutamina sintetasa plastídica, ha permitido avanzar en el esclarecimiento de la significación fisiológica exacta de dicha enzima en relación con la fotorrespiración y el estrés abiótico, haciendo uso de datos transcriptómicos y metabolómicos y análisis de la co-expresión de redes de genes. Entre las conclusiones más destacadas se incluyen la descripción de la existencia de una regulación coordinada de los genes fotorrespiratorios bajo condiciones de alta acumulación de NH_4^+; la inducción de rutas alternativas al ciclo GS2 / Fd-GOGAT para la reasimilación del NH_4^+; la interconexión de la fotorrespiración con el metabolismo central del carbono, del nitrógeno y metabolismo secundario; y el papel de la glutamina sintetasa plastídica en la respuesta al balance C / N y su relación con el metabolismo de la prolina y el estrés hídrico.

ABSTRACT

In this Doctoral Thesis it has been studied the nitrogen metabolism and its relationship with the photorespiration and abiotic stress in the model legume *Lotus japonicus*, from the point of view of the functional genomics. The availability of *Ljgln2-2* mutants, lacking completely of plastidic glutamine synthetase activity and protein, allowed to achieve a significant advance in the knowledge of the exact physiological significance of this enzyme in relation to the photorespiration and abiotic stress, making use of transcriptomic and metabolomic data and analysisi of co-expression networks. The most important conclusions of this Thesis are the following: description of the existence of a coordinate regulation of the photorespiratory genes in conditions of high accumulation of NH_4^+; the induction of alternative routes to the cycle GS2 / Fd-GOGAT for the reassimilation of the NH_4^+; the interconnection of the photorespiration with the central carbon metabolism, nitrogen metabolism and secondary metabolism; and the role of the plastidic glutamine synthetase in the response to the C / N balance and its relation with the proline metabolism and drought stress.

INTRODUCCIÓN

1. La asimilación de nitrógeno en las plantas

El nitrógeno es uno de los nutrientes más importantes para las plantas y que limita más poderosamente su crecimiento en los hábitats naturales. Esto es en parte debido a que de todos los nutrientes minerales, el nitrógeno es el que se requiere en mayores cantidades para la producción y el mantenimiento de sus células. Por ejemplo, una proteína contiene por término medio alrededor de un 12% de nitrógeno respecto a su peso seco. Igualmente, las plantas herbáceas contienen un alto porcentaje de nitrógeno (entre 1,5 a 4,5 % de su peso seco). De ahí que la disponibilidad de nitrógeno tenga una vital importancia para la supervivencia de las plantas y éstas hayan desarrollado diferentes mecanismos de regulación metabólicos para garantizar el mejor aprovechamiento de este elemento. Por otra parte, esto justifica también la alta demanda de fertilizantes nitrogenados a la que se recurre en la agricultura moderna con vistas a una mayor producción (se estima a nivel mundial un aporte de 85-90 millones de toneladas de fertilizantes nitrogenados a los suelos) (Good *et al*., 2004). Por estos motivos, el aporte de fertilizantes nitrogenados constituye uno de los principales costes económicos de la agricultura. Además de ello, hay que señalar que el uso exacerbado de fertilizantes nitrogenados se ha demostrado que puede traer consigo serios problemas medioambientales por la contaminación de suelos y el agua, que origina problemas de eutrofización, así como la contribución de los mismos al calentamiento global a través de la emisión de óxidos de nitrógeno como consecuencia de la desnitrificación. De ahí que el conocimiento del metabolismo del nitrógeno tenga no sólo una gran importancia básica para el estudio del crecimiento y la productividad de las plantas, sino también, una gran importancia aplicada dirigida a lograr una menor necesidad de fertilizantes nitrogenados para propiciar una agricultura más sostenible. No cabe duda que el conocimiento a nivel molecular de los finos mecanismos de regulación de la utilización de nitrógeno en las plantas puede tener importantes repercusiones en la agricultura y constituye una pieza clave para obtener plantas con una mejor eficiencia de la utilización del nitrógeno (NUE) (Hirel *et al*., 2007; Masclaux-Daubresse *et al*., 2010).

La utilización de nitrógeno por las plantas implica varias etapas, que incluyen el transporte de las diferentes fuentes nitrogenadas desde el exterior al interior celular; su posterior reducción y asimilación dentro de las células, y, también, la translocación o remobilización (reciclaje del nitrógeno) en el interior del vegetal (Monza y Márquez, 2004) (Figura 1).

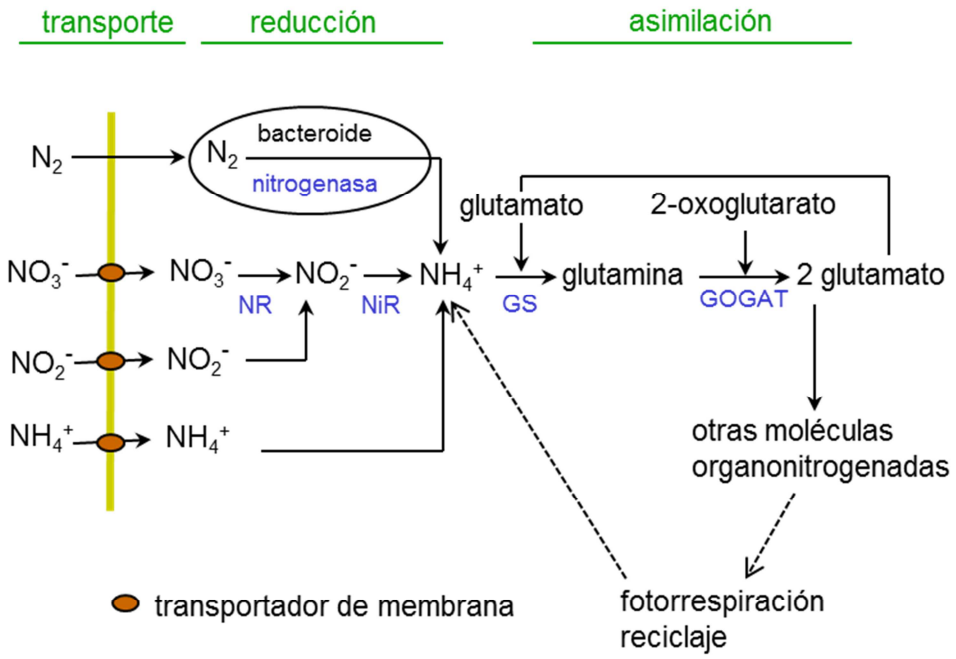

Figura 1. Esquema del proceso de asimilación de nitrógeno en las plantas. NR, nitrato reductasa; NiR, nitrito reductasa; GS, glutamina sintetasa; GOGAT, glutamato sintasa. Otros detalles en el texto.

La asimilación primaria de nitrógeno comprende la utilización de diferentes formas de nitrógeno inorgánico, fundamentalmente nitrato o amonio o una mezcla de ambos, dependiendo de la disponibilidad y de las especies vegetales (Forde y Clarkson, 1999; Andrews *et al.*, 2013). La nutrición mixta (nitrato más amonio) suele ser más efectiva para la mayoría de las plantas que la nutrición con nitrato solo, por diversas razones entre las que se incluye la compensación de los cambios de pH. La nutrición exclusiva con amonio es más rara en la naturaleza por las bajas concentraciones existentes de este ion, que raramente supera el valor de 50 μM, como consecuencia de la nitrificación, con lo cual este proceso sólo suele darse en suelos encharcados y en consecuencia anaeróbicos, o en suelos ácidos en los que la nitrificación no tiene lugar. Por este motivo sólo algunas especies han desarrollado las adaptaciones necesarias para la nutrición amoniacal. Para la mayoría de las especies el amonio origina problemas de toxicidad y han tenido que desarrollar mecanismos de destoxificación de este ion (Britto y Kronzucker, 2002). Alternativamente, algunas plantas como las leguminosas han desarrollado procesos simbióticos con bacterias que permiten la formación de estructuras especializadas llamadas nódulos donde la nitrogenasa bacteriana es capaz de desarrollar la fijación biológica del dinitrógeno atmosférico generando también amonio que es posteriormente asimilado. A continuación se describen someramente los diferentes procesos de asimilación de nitrato y amonio.

El nitrato es incorporado al interior celular mediante una serie de sistemas de transporte de alta o baja afinidad (HATS ó LATS respectivamente). Los procesos de transporte de nitrato han sido recientemente muy estudiados y existen excelentes revisiones al repecto que describen también el importante papel señalizador del nitrato en el crecimiento vegetal y en otros procesos como la defensa ante patógenos o la respuesta al estrés (Dechorgnat *et al.*, 2011; Fernández y Galván, 2008; Forde, 2000; Gojon *et al.*, 2011; Miller *et al.*, 2007; Orsel *et al.*, 2002; Wang *et al.*, 2012). Por lo general se han descrito dos familias de transportadores: la familia NRT1/PTR, que recientemente ha sido redenominada NPF (Leran *et al.*, 2013) y la familia NRT2, ambas ampliamente estudiadas. La familia NRT2 codifica para transportadores que contribuyen al sistema de alta afinidad, mientras que la familia NPF es la principal responsable de los sistemas de baja afinidad, aunque se conoce que algunos miembros de esta familia pueden mostrar o bien alta afinidad o bien una doble (alta y baja) afinidad. Una vez dentro de la célula el nitrato es reducido hasta amonio por la acción consecutiva de la nitrato reductasa (NR), que reduce el nitrato a nitrito a partir de NADH (EC 1.6.6.1) ó NAD(P)H (EC 1.6.6.2), seguido de la acción de la nitrito reductasa (NiR) (EC 1.7.7.1), que reduce el nitrito hasta amonio con 6 electrones suministrados por la ferredoxina reducida.

El amonio producido en las plantas puede ser resultante de varios procesos: a) reducción de nitrato; b) fijación simbiótica de dinitrógeno catalizada por la nitrogenasa (EC 1.18.6.1/EC 1.19.6.1); c) otros procesos capaces de generar amonio endógenamente en los vegetales tales como la fotorrespiración, el catabolismo de aminoácidos, o el metabolismo de los ureidos, amonio que ha de ser también muy eficientemente reasimilado (*asimilación secundaria de amonio*) ; d) el transporte directo de amonio desde el exterior. Se han descrito diferentes sistemas de transporte de alta o baja afinidad para el amonio, y que implican diferentes familias de transportadores (AMT1 ó AMT2) ampliamente investigados.

La asimilación de amonio constituye la etapa final del proceso asimilador de nitrógeno y consiste en la incorporación del amonio a esqueletos carbonados que se lleva a cabo, fundamentalmente, por la acción consecutiva de las enzimas glutamina sintetasa (GS, EC1.6.3.2) y glutamato sintasa (GOGAT). Esta ruta de asimilación de amonio se conoce con el nombre de ciclo GS-GOGAT, y ha sido ampliamente estudiada, existiendo muy buenas revisiones al respecto (e.g. Hirel y Lea, 2002; Lam *et al.*, 1996; Lea y Ireland, 1999, Masclaux-Daubresse *et al.*, 2010). La GS cataliza la incorporación del amonio a una molécula de glutamato mediante una amidación dependiente de la hidrólisis de ATP, produciendo glutamina. Esta enzima en las plantas viene codificada por una pequeña familia

de genes responsables de la existencia de distintos tipos de isoformas citosólicas (GS1) o plastídicas (GS2) en los distintos órganos de la planta. Por su parte, la GOGAT utiliza la glutamina formada junto con 2-oxoglutarato y dos equivalentes de reducción proporcionados bien por la ferredoxina reducida (Fd-GOGAT, EC 1.4.7.1) ó el NADH (NADH-GOGAT, EC 1.4.1.14), generando dos moléculas de glutamato, una de las cuales será utilizada por la GS, y la otra será el resultado neto del ciclo. Glutamina y glutamato son así los dos productos organonitrogenados primarios de la asimilación del nitrógeno, desde los cuales se distribuye el nitrógeno para la síntesis del resto de metabolitos y macromoléculas nitrogenadas del vegetal.

Tras su asimilación inicial en glutamina y glutamato, el nitrógeno es incorporado a otros aminoácidos mediante reacciones de transaminación catalizadas por las enzimas denominadas transaminasas, como la aspartato aminotransferasa que cataliza la transferencia reversible del grupo amino del glutamato al oxalacetato produciendo 2-oxoglutarato y aspartato, el cual constituye uno de los productos iniciales de la asimilación del nitrógeno, y es el precursor metabólico de la asparragina. La asparragina no sólo forma parte de las proteínas sino que también desempeña una importante función en el transporte y el almacenamiento del nitrógeno, como lo demuestra el hecho de que constituye uno de los compuestos nitrogenados más abundantes en el floema de las plantas, particularmente las leguminosas de clima templado como por ejemplo *Lotus japonicus* (Credali *et al.*, 2011, 2013). La asparragina es un aminoácido transportador de nitrógeno más económico que la glutamina en cuanto a gasto de carbono, lo cual es de gran importancia para la planta en situaciones de déficit de carbono. La síntesis de asparragina está catalizada por la enzima asparragina sintetasa (AS) (E.C. 6.3.5.4.) que está codificada por un número variable de genes dependiendo de las diferentes especies. Esta enzima cataliza la reacción de transferencia del grupo amido de la glutamina al aspartato para producir glutamato y asparragina, y también se discute que podría utilizar amonio cuando éste se haya a alta concentración, sirviendo así para destoxificarlo. La posterior acción de la asparraginasa (NSE, EC 3.5.1.1), es el principal mecanismo que posibilita la rotura de la asparragina translocada en el vegetal liberando aspartato y amonio, que ha de ser también eficientemente reasimilado por la ruta GS-GOGAT.

La enzima glutamato deshidrogenasa (GDH) (E.C. 1.4.1.2.) tiene también un papel fundamental en la homeostasis de glutamato en los vegetales pues cataliza la reacción reversible de aminación/desaminación del glutamato, lo que daría lugar a que esta enzima podría participar tanto en la síntesis como en el catabolismo de este aminoácido. El papel de esta enzima en el metabolismo del

glutamato en las plantas ha estado sujeto a una continua controversia ya que hace varias décadas se pensaba que la GDH catalizaba la aminación reductiva del 2-oxoglutarato sintetizando glutamato, constituyendo esta reacción la ruta de asimilación del amonio en las plantas. Sin embargo, las investigaciones posteriores demostraron que el glutamato se sintetizaba vía la acción combinada de GS y GOGAT, mientras que la GDH era responsable de la desaminación del glutamato. Más recientemente se ha vuelto a cuestionar el papel asimilador de la GDH puesto que se ha demostrado que la actividad de la GDH se incrementaba durante la senescencia y en condiciones de estrés, situaciones en las que aumentaba la concentración de amonio, lo que sugería que, en estas condiciones, la enzima podría actuar incorporando el amonio en aminoácidos y funcionando también como sistema de destoxificación del amonio (Forde y Lea, 2007; Fontaine *et al.,* 2012).

2. Interconexión entre el metabolismo del nitrógeno y la fotorrespiración

La fotorrespiración es un proceso resultante de la reacción de oxigenación de la Ribulosa-1,5-bisfofato-carboxilasa/oxigenasa (RUBISCO, EC 4.1.1.39), que tiene lugar en el mismo centro activo que la carboxilación, y que da lugar a una compleja ruta del metabolismo del carbono denominada ciclo C_2 o también ciclo del glicolato por implicar a este compuesto de dos átomos de carbono (para revisones recientes cf. Bauwe *et al.,* 2010; Peterhansel *et al.,* 2010). Aunque la afinidad de la RUBISCO por el CO_2 es mucho más alta que por el oxígeno, el hecho de que la concentración atmosférica de oxígeno (21%) sea mucho mayor que la de dióxido de carbono (0,03%) implica que ambas reacciones contribuyen de forma importante al consumo de ribulosa-1,5- bisfosfato en condiciones normales. La magnitud de la fotorrespiración medida como intercambio gaseoso varía entre las diferentes especies de plantas, desde valores bajos de las plantas C4 a los altos valores de las plantas C3 (25-50% de la tasa fotosintética). La oxigenación de la ribulosa 1,5-bisfosfato produce fosfoglicolato y una molécula de fosfoglicerato. El metabolismo del fosfoglicolato constituye así una desviación del carbono del ciclo de Calvin y se estima que reduce la asimilación neta del CO_2 hasta una tercera parte.

Durante muchos años, los estudios sobre el metabolismo fotorrespiratorio se centraron en las reacciones que implicaban al carbono. Llegó a ser evidente que la principal función del ciclo C_2 era recuperar para la fotosíntesis tres de cada cuatro átomos de carbono que eran inicialmente desviados como fosfoglicolato, con la ventaja adicional de eliminar así el fosfoglicolato que es un compuesto fuertemente tóxico. En última instancia, este ciclo devolvía carbono al

cloroplasto como 3-fosfoglicerato. El cuarto carbono se liberaría como CO_2 y debería ser fijado (Figura 2).

Este ciclo C_2 implica reacciones que tienen lugar en tres orgánulos de la célula. En primer lugar, el flujo de carbono en el ciclo oxidativo se inicia en los cloroplastos, después, sigue en los peroxisomas donde ocurre la conversión del glicolato en glioxilato y de éste a glicina, y por último, implica también a la mitocondria donde dos moléculas de glicina se transforman en una molécula de serina, CO_2 y NH_3. Por tanto, la reacción que tiene lugar en la mitocondria es la principal responsable de la liberación del CO_2.

$$2\ \text{Glicina} + H_2O \longrightarrow \text{Serina} + CO_2 + NH_3 + 2H^+ + 2e^-$$

Como puede observarse, la liberación de dióxido de carbono va acompañada de la liberación simultánea y estequiométrica de amonio, el cual debe ser eficientemente asimilado para evitar la acumulación masiva de este compuesto dado su toxicidad. Keys *et al.* (1978) mostraron la importancia de este nitrógeno como un elemento crucial del metabolismo fotorrespiratorio. Basándose en cálculos aproximados de la fotorrespiración en hojas de trigo, se determinó que la pérdida de amonio podría sobrepasar en 10 veces la tasa calculada de asimilación primaria de NH_3 proveniente de la reducción de NO_3^-. Según los resultados obtenidos, se propuso que la reasimilación del amonio fotorrespiratorio era también un proceso crucial a considerar en la asimilación de nitrógeno.

Así, se comprobó que el amonio liberado por el ciclo C_2 es rápidamente reasimilado mediante el ciclo de la GS-GOGAT. Dos tipos de evidencias fundamentales permitieron a Keys *et al.* (1978) llegar a esta conclusión: 1) aunque las mitocondrias contienen GDH, no eran capaces de reasimilar amonio; 2) la presencia de metionina sulfoximina (MSX), conocido inhibidor de la GS, bloqueaba rápidamente la reasimilación del NH_3, causando una rápida y alta acumulación. Keys *et al.* (1978) propusieron inicialmente que sería la GS citosólica (GS1) la que llevaría a cabo la reasimilación del amonio fotorrespiratorio dado que el amonio se producía en la mitocondria. Posteriormente se demostró que no era la GS1 sino la GS2 la isoforma encargada de asimilar el amonio generado en el ciclo fotorrespiratorio. Para ello fue clave la obtención de mutantes deficientes en la isoforma cloroplástica de GS de cebada (Wallsgrove *et al.*, 1987) y que carecían de la capacidad de reasimilar amonio. La participación inequívoca de la Fd-GOGAT en la reasimilación de amonio fotorrespiratorio había sido ya anteriormente puesta de manifiesto mediante mutantes en *Arabidopsis* (Somerville y Ogren, 1980) y pudo ratificarse también en cebada (Kendall *et al.*, 1986).

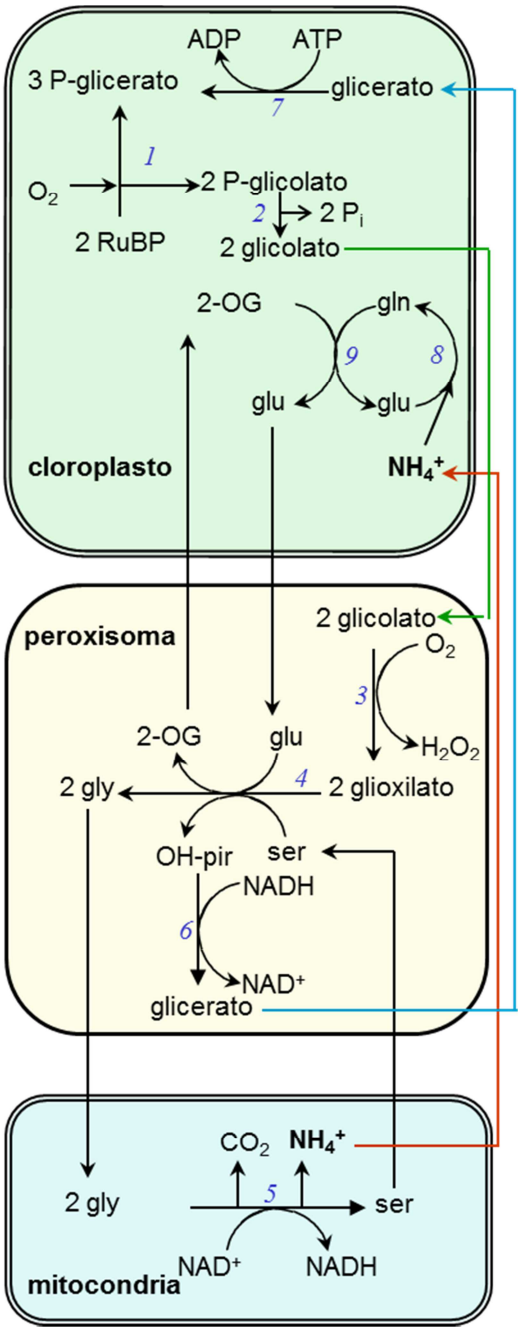

Figura 2. Esquema del ciclo C_2 ó ciclo fotorrespiratorio del nitrógeno (modificado de Monza y Márquez, 2004). *1, RUBISCO; 2, fosfoglicolato fosfatasa; 3, glicolato oxidasa; 4, glioxilato transaminasas; 5, complejo multienzimático glicina descarboxilasa / serina hidroximetiltransferasa; 6, hidroxipiruvato reductasa; 7, glicerato kinasa; 8, glutamina sintetasa; 9, glutamato sintasa. RuBP, Ribulosa-1,5-bisfato*

En definitiva, gracias al uso de inhibidores químicos que bloquean el ciclo de la GS-GOGAT o que interfieren con otras enzimas de reacciones específicas del ciclo oxidativo C_2, y también al uso de mutantes fotorrespiratorios, se puede afirmar que la conversión de glicina a serina es la mayor fuente de NH_3 en hojas que fotorrespiran activamente y que la GS cloroplástica es la enzima responsable de su reasimilación.

Edwards *et al.* (1990) demostraron que la GS1 y GS2 están compartimentalizadas en distintos tipos de células, no existiendo GS1 en las células en las que se lleva a cabo la fotorrespiración, lo que explicaría este papel crucial de la GS2 en la reasimilación del amonio fotorrespiratorio y que la GS1 no realice esta labor. De hecho, Oliveira *et al*. (2002) desarrollaron una expresión ectópica de GS1 en células del mesófilo de hojas de tabaco, y demostraron que esta expresión producía alteraciones en el proceso fotorrespiratorio que se evidenciaban por una disminución del nivel de amonio, elevados niveles de intermediarios fotorrespiratorios y un incremento de los niveles de glutamato, producto de la asimilación del amonio procedente de la fotorrespiración. Por tanto, esta expresión ectópica de GS1 en células de las hojas estaría proporcionando una ruta complementaria o alternativa de la GS2 en la reasimilación del amonio fotorrespiratorio.

Por otro lado, el flujo continuo de carbono en el ciclo fotorrespiratorio antes mencionado depende de un aporte suficiente de donadores de grupos aminos para la transaminación del glioxilato a glicina en los peroxisomas. La producción de los aminoácidos necesarios que actúen de donadores de los grupos aminos depende y está íntimamente ligada también a la reincorporación del amonio derivado de la fotorrespiración (Keys *et al.* 1978). Igualmente, el flujo de metabolitos entre distintos orgánulos, necesario para el funcionamiento del ciclo C2, requiere de todo un amplio elenco de transportadores y lanzaderas de aminoácidos y ácidos orgánicos (Douce y Neuburger, 1999; Peterhansel *et al.*, 2010).

3. Interconexión entre el metabolismo del nitrógeno y el estrés hídrico

El crecimiento de las plantas se ve limitado por diferentes factores, entre ellos el estrés osmótico generado por déficit hídrico, bajas temperaturas, o altas concentraciones de sal. En condiciones de sequía, el potencial hídrico del suelo se hace más negativo, por lo que las plantas deben disminuir este potencial para mantener el gradiente necesario para la absorción de agua. La célula vegetal puede disminuir su potencial hídrico por la acumulación de solutos, o por pérdida de agua. En suelos en condiciones de sequía, un mecanismo que tienen las plantas para evitar la disminución del potencial hídrico es la acumulación de solutos orgánicos o inorgánicos. Los solutos orgánicos que se acumulan en el citoplasma en condiciones de estrés osmótico se denominan osmolitos compatibles, mientras que los iones inorgánicos que se acumulan en vacuola y participan también en el mantenimiento del potencial hídrico se conocen como osmolitos celulares. Entre los osmolitos compatibles más frecuentes se

encuentran polioles, sacarosa, glicín-betaína y prolina, que pueden acumularse a concentraciones elevadas, de manera que evitan la disminución del potencial hídrico sin interferir con el funcionamiento celular (Díaz *et al.*, 2004).

Por otro lado, el estrés osmótico, como todo estrés abiótico, induce en las plantas la formación de especies reactivas de oxígeno (ROS), como el radical superóxido, el oxígeno singlete, el peróxido de hidrógeno y el radical hidroxilo, que producen daños a fosfolípidos, ácidos nucelicos y proteínas, entre otras moléculas. La formación de ROS también la inducen el ozono, el óxido nítrico, óxido nitroso y óxido sulfuroso y cualquiera sea el desencadenante, el estrés oxidativo se genera cuando la producción de ROS por la planta es mayor a la capacidad de eliminarlas. Algunos de los osmolitos sintetizados por la planta pueden funcionar como capturadores de ROS, protegiendo a la célula del estrés oxidativo. Esos osmolitos constituyen una defensa antioxidante no enzimática, a diferencia de la defensa enzimática en la que participan la superóxido dismutasa y peroxidasas, entre otras enzimas. Por este motivo, la síntesis de osmolitos nitrogenados y glutatión juega un papel determinante en la tolerancia al estrés abiótico de las plantas a través del mantenimiento del potencial hídrico, la destoxificación de ROS y la regulación del pH celular. En otros trabajos pueden encontrarse más detalles sobre este particular (Díaz *et al.*, 2004).

En diferentes especies se ha comprobado que la asimilación de nitrógeno, y, en particular, la GS, juegan un papel determinante en relación con el estrés abiótico y, en concreto, en el estrés hídrico (Yousfi *et al.*, 2012). De hecho, se ha descrito un claro papel de la GS citosólica en la producción de prolina (Brugiére *et al.*, 1999). Igualmente se demostrado que la sobreexpresión de GS cloroplástica en tabaco y arroz resulta en una mayor tolerancia a la fotooxidación (Kozaki y Takeba, 1996) y al estrés salino (Hoshida *et al.*, 2000). El trabajo de Kozaki y Takeba (1996) es particularmente interesante porque enfatiza la posible participación de la fotorrespiración y la GS2 en la protección frente al estrés abiótico, como un mecanismo para prevenir la sobrereducción del estroma, y por ende la fotoinhibición, mecanismo sugerido también por otros autores para diferentes situaciones de estrés y también para las condiciones normales de vida de las plantas (Douce y Neuburger, 1999; Maurino y Peterhansel, 2010; Osmond *et al.*, 1997; Wingler *et al.*, 2000). De hecho, se ha comprobado que la transferencia de equivalentes de reducción puede ser importante para la asimilación de nitrato (Rachmilevitch *et al.*, 2004) y que que la fotorrespiración puede ser importante para evitar la supresión de la reparación del fotosistema II que ha sido fotodañado (Takahashi *et al.*, 2007). Por otra parte, la fotorrespiración contribuye también a la homeostasis redox de las plantas a través de la producción de H_2O_2 e implicación de los piridín nucleótidos (Foyer *et al.*,

2009). Los estudios con mutantes de cebada deficientes en GS2 sugieren que el equilibrio redox en los diferentes compartimentos celulares tiene mucho que ver con los efectos adversos de la deficiencia en el metabolismo fotorrespiratorio (Igamberdiev *et al.*, 2001). No obstante hay que mencionar también que existen autores para los que es difícil aceptar que la fotorrespiración sea un proceso esencial para proteger las plantas frente al exceso de luz (Keys, 2006).

4. Estudios previos sobre la asimilación de nitrógeno y su interrelación con la fotorrespiración y el estrés hídrico en la leguminosa modelo *Lotus japonicus*

La planta objeto de los estudios del presente trabajo es la leguminosa modelo *Lotus japonicus*. Los estudios con leguminosas modelo surgieron a raíz de que las investigaciones en biología molecular, genética y genómica de la mayoría de las leguminosas de interés agrícola se veían dificultados por sus largos tamaños de genoma y otras desventajas (poliploidía, dificultad en su transfpormación y regeneración, escaso número de semillas y plántulas, duplicaciones génicas, largos tiempos de generación, etc.). Dos han sido las leguminosas modelo más ampliamente utilizadas hasta la fecha: *Medicago truncatula*, que posee nódulos indeterminados, y *Lotus japonicus*, como modelo de leguminosa con nódulos determinados, a raíz de la propuesta efectuada por Handberg y Stougaard (1992). Se trata de una planta diploide, de pequeño tamaño, que se reproduce por auto-fertilización o por esquejes, con un genoma relativamente pequeño (450 Mbp) en base a 12 cromosomas, que presenta grandes facilidades de cultivo y producción de semillas, y que se puede regenerar desde callos siendo posible la transformación con *Agrobacterium tumefaciens.* Desde entonces han sido muchas las herramientas moleculares que se han desarrollado para el trabajo con esta planta, incluyendo la secuenciación de su genoma llevada a cabo fundamentalmente por el instituto Kazusa de Japón, y que ha generado la base de datos 2.5 que está siendo ampliamente utilizada (http://www.kazusa.or.jp/lotus). Igualmente se han desarrollado otras herramientas de genómica funcional como secuenciación de ESTs, chips de DNA para análisis del transcriptoma, basados en dichas secuencias, proteómica, metabolómica a través de GC-MS, mapeo génico y nuevas herramientas de mutagénesis para genética inversa tales como el TILLING (targeted induced local lesions in genomes) (Perry *et al.*, 2003) y más recientemente la mutagénesis con transposones LORE1 (Urbansky *et al.* 2012 ; Fukai *et al.* 2012). Diferentes trabajos detallan las enormes posibilidades que ofrece la leguminosa modelo *Lotus japonicus* para la investigación y los principales avances logrados merced al trabajo con esta leguminosa, en su mayor parte relativos a sus procesos

simbióticos, bien con la bacteria *Mesorhizobium loti* para el proceso de la fijación biológica del nitrógeno o mediante micorrización (cf. Madsen *et al.*, 2010; Márquez, 2005; Udvardi *et al.*, 2005; Sato y Tabata, 2005; Stacey *et al.*, 2006).

Nuestro grupo de trabajo ha venido trabajando con *L. japonicus* desde hace unos 20 años, poco después de la propuesta de esta planta como leguminosa modelo y merced a distintos proyectos nacionales e internacionales que se han ido sucediendo en el tiempo. Los primeros trabajos del grupo se centraron en la caracterización de los sistemas de reducción de nitrato y nitrito en *L. japonicus* (Márquez *et al.*, 2004; Márquez *et al*., 2005a; Márquez *et al.*, 2005b; Orea *et al.*, 2001; Orea *et al.*, 2005a; Orea *et al.*, 2005b; Pajuelo *et al.*, 2002), que se suman también a los producidos por otros autores (Harrison *et al.*, 2004; Prosser *et al.*, 2006). Otros trabajos más recientes han caracterizado el metabolismo de la asparragina en esta planta, con especial énfasis en el estudio de la asparraginasa (Credali *et al.*, 2011; Credali *et al.*, 2013), que completan otros estudios anteriores que pusieron de manifiesto la importancia de la asparragina para la translocación de nitrógeno en esta planta (Waterhouse *et al.*, 1996). Sin embargo, los trabajos más relacionados con esta Tesis son los concernientes a la caracterización de mutantes fotorrespiratorios de *L. japonicus* deficientes en GS2. Este tipo de mutantes se obtuvieron (Orea *et al.*, 2002; Márquez *et al.*, 2005a) tras un escrutinio de semillas mutagenizadas con etil metano sulfonato (EMS) y se identificaron por su capacidad de crecimiento normal, similar al silvestre (WT), en condiciones de alto CO_2 (0,7 % v/v), donde la fotorrespiración está suprimida, pero que, a diferencia con las plantas silvestres mostraban daños importantes (clorosis y necrosis de las hojas) cuando se las transfería a una atmósfera de aire normal, que son condiciones de fotorrespiración activa (Figura 3). Las plantas pueden recuperarse perfectamente volviéndolas a la atmósfera de alto CO_2.

Este mismo procedimiento fue el que inicialmente se diseñó para la búsqueda de mutantes fotorrespiratorios en *Arabidopsis* (Somerville y Ogren, 1979; 1982), existiendo ya disponibles en *Arabidopsis* un amplio catálogo de mutantes afectados en distintas etapas del ciclo fotorrespiratorio C2 (Timm y Bauwe, 2013). Igualmente se han descrito también mutantes fotorrespiratorios en otras especies como cebada y guisante (Blackwell *et al.*, 1988; Keys y Leegood, 2002). Sin embargo, mutantes específicamente deficientes en GS2 sólo han podido obtenerse en cebada, y fueron fundamentales, como se explicó anteriormente, para demostrar la implicación específica de la GS2 en la reasimilación del amonio fotorrespiratorio. Por esta razón, los mutantes obtenidos en nuestro grupo en *L. japonicus*, específicamente afectados en GS2,

son la primera leguminosa en la que se consiguen y permiten explorar la significación fisiológica precisa de esta isoforma particular de GS.

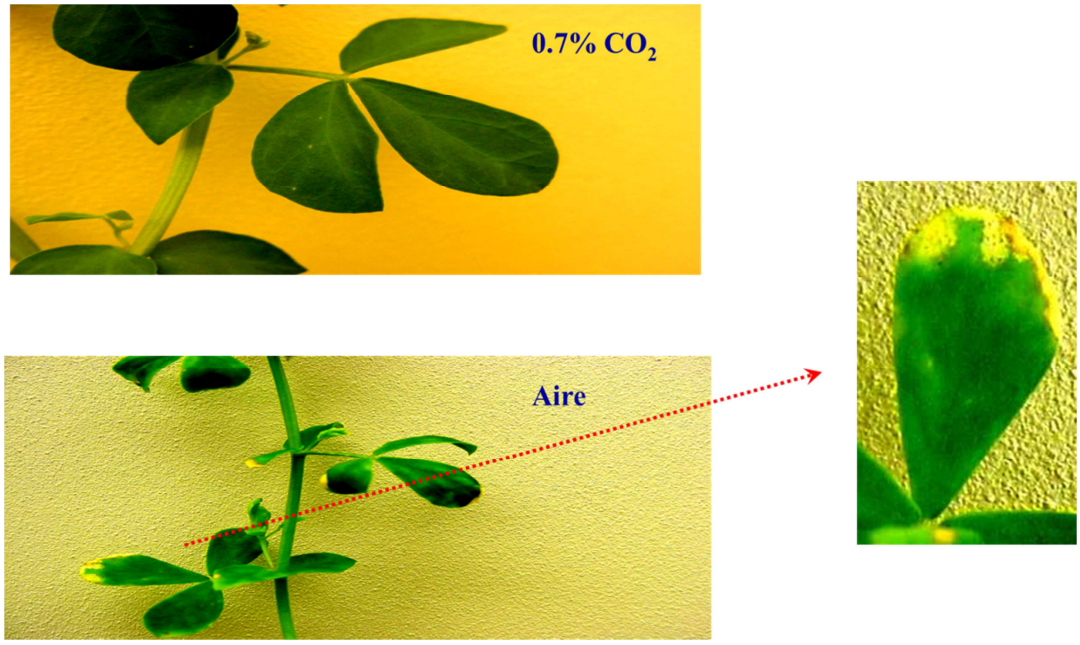

Figura 3. Fenotipo de sensibilidad a aire de los mutantes fotorrespiratorios de *L. japonicus* deficientes en GS2. Las plantas muestran un aspecto normal cuando se cultivan en una atmósfera enriquecida en CO_2 pero exhiben claros síntomas de clorosis y necrosis de las hojas al cabo de 7-10 días de la transferencia a aire normal, comenzando por las hojas más jóvenes.

En un principio se obtuvieron dos mutantes, inicialmente denominados *Ljpr1* y *Ljpr2* que fueron posteriormente caracterizados demostrándose que poseían niveles normales de GS1 y que eran alélicos y mostraban una herencia mendeliana debida a un solo carácter recesivo (Orea *et al.*, 2002; Márquez *et al.*, 2005a). Mediante mapeo génico pudo comprobarse en *L. japonicus* la existencia de un único gen *GLN2* codificante para la GS2 (Sandal *et al.*, 2006). Posteriormente, se constató que ambos mutantes estaban específicamente afectados en la región codificante del gen para GS2 (*LjGLN2*), por lo que dichos mutantes se denominaron respectivamente *Ljgln2-1* y *Ljgln2-2* (Betti *et al.*, 2006), mostrando respectivamente mutaciones puntuales responsables de las sustituciones G85R y L278H en el polipéptido correspondiente. La producción de las proteínas recombinantes mutantes en *E. coli* permitió comprobar que ambas mutaciones anulan por completo la actividad GS2 pero afectan de manera diferente a la estabilidad del polipéptido para GS2 (Betti *et al.*, 2006). En el caso del mutante *Ljgln2-1* se pueden detectar pequeños niveles del polipéptido para GS2 en hojas, raíces y nódulos de las plantas mutantes, mientras que el mutante *Ljgln2-2* carece por completo de proteína (Betti *et al.*, 2006; García-Calderón *et al.*, 2012). Este mutante *Ljgln2-2* es el que se ha utilizado fundamentalmente en

esta Tesis. Otros estudios anteriores llevados a cabo con estos mutantes han permitido esclarecer la significación exacta de la GS2 para la nodulación de las plantas (García-Calderón *et al.,* 2012) demostrándose una clara vinculación de esta enzima en el balance C/N de las plantas de *L. japonicus*, fundamental para este proceso. Por otra parte, también se han utilizado anteriormente estos mutantes para investigar su respuesta a sequía, demostrándose una clara implicación de la GS2 en la producción de prolina dado que los mutantes mostraban una menor acumulación de prolina en respuesta al estrés hídrico, acompañado de profundas alteraciones del transcriptoma de las plantas indicativa de que están percibiendo una mayor situación de estrés y que se traduce también en una menor capacidad de rehidratación de las mismas tras la sequía (Díaz *et al.*, 2010). Otros trabajos relativos a la GS de *L. japonicus* llevados a cabo por otros autores son escasos y conciernen fundamentalmente a la GS1 (Limami *et al.*, 1999; Harrison *et al.*, 2000; 2003; Ortega *et al.* 2004; Suárez *et al.*, 2003).

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OBJETIVOS

La disponibilidad de mutantes *Ljgln2-2*, carentes por completo de actividad y proteína GS2, permitía avanzar en el esclarecimiento de la significación fisiológica exacta de la glutamina sintetasa plastídica en relación con la fotorrespiración, nutrición nitrogenada y estrés hídrico en *L. japonicus* abordándose en esta Tesis los siguientes objetivos:

1. Obtención y análisis comparado del transcriptoma y metaboloma de las plantas silvestres y mutantes *Ljgln2-2* de *L. japonicus* en condiciones de fotorrespiración suprimida (alto CO_2) y fotorrespiración activa (aire normal), para identificar los genes y metabolitos que se ven modulados común o diferencialmente en ambos genotipos, y establecer conclusiones sobre la posible implicación de la fotorrespiración y/o la acumulación de amonio fotorrespiratorio en el metabolismo primario y secundario de las plantas. Este objetivo se desarrolla fundamentalmente en la Publicación 1:

Publicación 1:

Transcriptomic and Metabolic changes associated with photorespiratory ammonium accumulation in the model legume *Lotus japonicus.* **Pérez-Delgado CM**, García-Calderón M, Sánchez DH, Udvardi MK, Kopka J, Márquez AJ, Betti M (2013) *Plant Physiology* **162**, 1834-1848.

2. Análisis de la posible inducción de enzimas alternativas a la GS2 para la reasimilación de amonio fotorrespiratorio. Para este objetivo se efectuó el análisis de los principales genes y enzimas implicadas en la reasimilación de amonio tanto en condiciones de fotorrespiración suprimida (alto CO_2) como fotorrespiración activa (aire normal) (Publicación 2):

Publicación 2:
Alternative reassimilation of photorespiratory ammonium in *Lotus japonicus*.
Pérez-Delgado CM, García-Calderón M, Márquez AJ, Betti M. Unpublished.

3. Estudio comparado de la influencia de la fuente nitrogenada sobre el transcriptoma de plantas WT y mutantes *Ljgln2-2* en condiciones de fotorrespiración suprimida (alto CO_2) y fotorrespiración activa (aire normal), que se muestra en la Publicación 3.

Publicación 3:
Transcriptomics of leaves of *Lotus japonicus* plants grown under different nitrogen regimes. Analysis of primary and secondary nitrogen assimilation.

Pérez-Delgado CM, Moyano TC, García-Calderón M, Márquez AJ, Gutiérrez RA, Betti M. Unpublished.

4. Estudio comparado de la transcriptómica de los genes modulados por la fotorrespiración y la sequía en plantas silvestres y mutantes *Ljgln2-2* (Publicación 4), seguido por un análisis específico de la transcriptómica de la sequía en condiciones de fotorrespiración activa (aire normal) (Publicación 5).

Publicación 4:
Abiotic stress in Lotus: aluminum and drought.
Pal'ove-Balang, Betti M, Díaz P, **Pérez-Delgado CM**, García-Calderón M, Monza J, Márquez AJ (2014). En: *Molecular approaches in Plant Abiotic Stress* (Gaur RK, Sharma P, eds). CRC Press, Boca Raton, Florida, USA, pp. 291-303.
Cf. Apartado titulado "Drought stress"

Publicación 5:
Cellular Stress Following Water Deprivation in the model legume *Lostus japonicus*.
Betti M, **Pérez-Delgado C**, García-Calderón M, Díaz P, Monza J, Márquez AJ (2012) *Cells* **1**, 1089-1106.

Otras Publicaciones:

En este apartado de la Tesis se muestran también otras publicaciones directamente relacionadas con la misma donde se incluyen resultados de los objetivos de esta Tesis en los apartados que se mencionan a continuación.

Publicación 6:
Glutamine synthetase in legumes: recent advances in enzyme structure and functional genomics.
Betti M, García-Calderón M, **Pérez-Delgado CM**, Credali A, Estivill G, Galván F, Vega JM, Márquez AJ (2012) *International Journal of Molecular Sciences* **13**, 7994-8024.
Cf. Apartados 2.2.1 (objetivo 1), 2.2.2 (objetivo 2), 2.2.4 (objetivo 4) y 2.2.5 (integración de todos los datos de los objetivos 1,2,3 y 4)

Publicación 7:
Reassimilation of Ammonium in *Lotus japonicus*

Betti M, García-Calderón M, **Pérez-Delgado CM**[*], Credali A, Pal'ove-Balang P, Estivill G, Repčák M, Vega JM, Galván F, Márquez AJ. *Journal of Experimental Botany* (sometido a publicación) [*] co-primera autora
Cf. Apartado "photorespiratory ammonium reassimilation" (objetivos 1 y 2)

Publicación 8:
Genes for ammonium assimilation.
Pérez-Delgado CM, García-Calderón M, Credali A, Vega JM, Betti M, Márquez AJ (2014) En: *The Lotus japonicus genome* (Tabata S, Stougaard J, eds), Springer (en prensa).
En este artículo se incluye un estudio genómico de los diferentes genes implicados en la asimilación de amonio en *L. japonicus* y sus niveles basales de expresión en distintos tejidos.
(objetivo 2)

Publicación 9:
A *Lotus japonicus* mutant deficient in nitrate uptake is also affected in the nitrate response to nodulation.
Pal'ove-Balang P, García-Calderón M, **Pérez-Delgado CM***, Pávlovin J, Betti M, Márquez AJ (2014) *Plant Biology* doi:10.1111/plb.12169. [*]co-primera autora

En este artículo se muestran los datos de niveles de expresión de un amplio número de genes implicados en el transporte, reducción y asimilación de nitrato, junto con otros posibles genes reguladores, obtenidos para plantas silvestres de *Lotus japonicus*, como complemento de los datos ilustrados en el artículo 3 (objetivo 3); estos datos se muestran en comparación con los datos obtenidos para el mutante *Ljclo1* deficiente en el transporte de nitrato.

PUBLICACIONES

Publicación 1.

Transcriptomic and Metabolic changes associated with photorespiratory ammonium accumulation in the model legume *Lotus japonicus.*

Pérez-Delgado CM, García-Calderón M, Sánchez DH, Udvardi MK, Kopka J, Márquez AJ, Betti M (2013) *Plant Physiology* 162, 1834-1848.

ABSTRACT

The transcriptomic and metabolic consequences of the lack of plastidic glutamine synthetase in the model legume *Lotus japonicus* were investigated. WT and mutant plants lacking of the plastidic isoform of glutamine synthetase were grown in conditions that suppress photorespiration (0.7% v/v CO_2) and then transferred for different lengths of time to photorespiratory conditions (normal air, 0.04% v/v CO_2). Transcript and metabolite levels were determined at the different time points considered. Under photorespiratory active conditions the mutant accumulated high levels of ammonium followed by its subsequent decline. A coordinate repression of the photorespiratory genes was observed in the mutant background. This was part of a greater modulation of the transcriptome, especially in the mutant, that was paralleled by changes in the levels of several key metabolites. The data obtained for the mutant represent the first direct experimental evidence for a coordinate regulation of photorespiratory genes over time. Metabolomic analysis demonstrated that mutant plants under active photorespiratory conditions accumulated high levels of several amino acids and organic acids, including intermediates of the Krebs cycle. An increase in glutamine levels was also detected in the mutant, that was paralleled by an increase in cytosolic GS1 gene transcription and activity levels. The global panoramic of the transcripts and metabolites that changed in *L. japonicus* plants during the transfer from photorespiration-suppressed to photorespiration-active conditions highlighted the link between photorespiration and several other cellular processes, including central carbon metabolism, amino acid metabolism and secondary metabolism.

INTRODUCTION

Plant photorespiration is caused by the light-dependent uptake of oxygen and is concomitant with the release of CO_2 and ammonia. The first step of photorespiration takes place in the chloroplast, where ribulose-1,5-bisphosphate-carboxylase/oxygenase (RUBISCO, EC 4.1.1.39) oxygenates a molecule of ribulose-1,5-bisphosphate to give one molecule of 3-phosphoglycerate and one of 2-phosphoglycolate. The latter molecule enters in a complex pathway, the C_2 cycle that encompasses three compartments: chloroplasts, mitochondria and peroxisomes. The cycle serves as a carbon recovery system by transforming 2-phosphoglycolate into 3-phosphoglycerate, that goes back to the Calvin cycle (Maurino and Peterhansel, 2010). Photorespiration also generates a high flux of ammonium that, at least in C3 plants, can be 10 times higher than the originated from nitrate reduction (Hirel et al., 2007). This ammonium is then reassimilated by the plastidic isoform of glutamine synthetase (GS_2).

Although the recovery of the carbon equivalents diverted from photosynthetic metabolism by the oxygenase activity of RUBISCO is probably the main function of photorespiratory metabolism, other roles have been proposed such as the dissipation of excessive reducing power in the chloroplast as a consequence of high light, drought or salt stress (Kozaki and Takeba, 1996; Hoshida et al., 2000; Maurino and Peterhansel, 2010). Photorespiration is also needed in order to produce metabolites for other pathways such as serine and glycine, with the latter that also serves as substrate for glutathione biosynthesis (Wingler et al., 2000). On the other hand, impairment of the photorespiratory cycle leads to increased photoinhibition of photosystem II through inhibition of the synthesis of the D1 protein (Takahashi et al., 2007).

Several of the steps of the photorespiratory pathway that were first defined through classical biochemical experiments were later confirmed by the isolation of photorespiratory mutants (Keys and Leegood, 2002). A mutant screening that was first devised to isolate photorespiratory mutants from Arabidopsis was based on the fact that photorespiratory mutants are conditionally lethal. Mutant plants grow normally under a CO_2-enriched atmosphere (>0.2%) where photorespiration is suppressed. However, when such mutants are transferred to normal CO_2 conditions they show various stress symptoms. Once the photorespiratory mutants have been identified the plants can be transferred back to CO_2-enriched atmosphere, permitting their recovery. Several of these mutants accumulated the substrate of the reaction of the mutated enzyme (Wingler et al., 2000), indicating that the photorespiratory cycle was active, albeit impaired, in

the mutant plants. This approach has been applied successfully to isolate photorespiratory mutants from several other species like barley and pea (Wingler et al., 2000; Foyer et al., 2009). The genetic analysis of such mutants allowed the identification of the genes involved in photorespiratory metabolism. Knockdown approaches in other species like tobacco also helped in the characterisation of the photorespiratory genes (Ferrario-Méry et al., 2000; 2002).

The photorespiratory cycle has an important impact over several other metabolic processes. First of all, mutants with reduced activity of photorespiratory enzymes often showed reduced rates of CO_2 assimilation. This can be caused either by depletion of Calvin cycle metabolites, by a decline of the nitrogen status of the leaf or by inhibition of Calvin cycle activities due to the accumulation of photorespiratory metabolites (Wingler et al., 2000). In addition, several important pathways like nitrogen assimilation, respiration, one-carbon metabolism, purine biosynthesis (Bauwe et al., 2010) and redox signalling (Foyer et al., 2009) interact in different ways with photorespiration. Despite of the impact of photorespiration on several aspects of plant metabolism and growth, the enzymes involved in this pathway have been completely identified only recently (Reumann and Weber, 2006). Moreover, there is little information about the transcriptional response of photorespiratory genes to environmental stimuli and if they may be regulated in a coordinate way (Foyer et al., 2009).

In our laboratory, the first photorespiratory mutants of legumes were isolated from the model legume *Lotus japonicus* (Orea et al., 2002; Márquez, 2005; Márquez et al., 2005). These mutants, deficient in plastidic GS_2, were further characterised at the molecular level (Betti et al., 2006) as well as nodule function (García-Calderón et al., 2012). Under CO_2-enriched atmosphere, the mutants did not show any visible phenotype and only a slightly lower growth rate (Orea et al., 2002). However, the mutants accumulated high levels of ammonium when transferred from CO_2-enriched to normal CO_2 atmosphere and showed severe stress symptoms when grown for long periods of time under these conditions. One of the mutants, named *Ljgln2-2*, was affected in its ability to recover after drought stress and presented a distinctive transcriptional response to water deprivation, demonstrating a novel link between plastidic GS_2 and drought-induced proline accumulation (Díaz et al., 2010). In this work, we carried out a comparative study of the response to active photorespiration in WT and *Ljgln2-2* mutant plants that were previously grown under conditions where the rate of photorespiration is very low (0.7% v/v CO_2; Orea et al., 2002). After the transfer to active photorespiratory conditions, a concerted regulation of the photorespiratory genes, together with massive transcriptomic changes and a

perturbation of central metabolism were observed in the case of the *Ljgln2-2* mutant. Interestingly, most changes in transcript and metabolite levels were either parallel or opposite to the ammonium ones. The data presented in this paper provide novel information on the regulation of photorespiration and on the interaction between the photorespiratory pathway and the central metabolism of legumes.

RESULTS AND DISCUSSION

Kinetics of ammonium accumulation in the *Ljgln2-2* mutant

Previous studies showed that photorespiratory mutants of *L. japonicus* that are deficient in plastidic GS_2 accumulated ammonium at least during the first 24 hours after exposure to normal CO_2 atmosphere (Orea et al., 2002). We aimed now to study the time-course process of ammonium accumulation and to address the effect of an impaired photorespiratory cycle on an extended time-scale. WT plants and the plastidic GS_2 mutant *Ljgln2-2* were grown under high CO_2 atmosphere (where photorespiration is very low) and then transferred to normal CO_2 atmosphere (normal photorespiration) for different periods of time up to 10 days. Free ammonium levels were determined in the sampled leaf tissue (Fig. 1). Not surprisingly, ammonium content in the WT leaves was extremely low over the time span of the experiment, indicating that the photorespiratory ammonium was efficiently reassimilated by GS_2. However, the *Ljgln2-2* mutant showed an almost 20-fold increase in NH_4^+ levels reaching a maximum of about 60 μmol $NH_4^+ \cdot g\ FW^{-1}$ after 3 days, a content about 150 times higher than that of the WT.

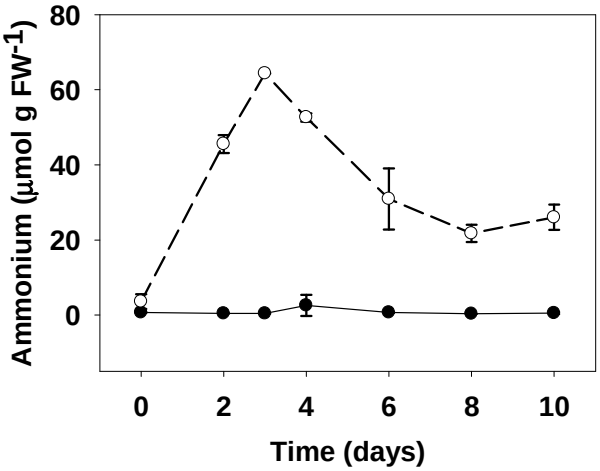

Figure 1. Ammonium content in leaves of WT and *Ljgln2-2* mutant plants. Free ammonium was determined in plant leaves at different times of the transfer from high CO_2 (time zero) to normal CO_2 conditions for the indicated periods of time. WT: black dots, solid line, *Ljgln2-2* mutant: white dots, dashed line. Data are the mean ± S.D. of three independent biological replicates. At some time points the error bars are too small to be noticed.

Interestingly, the ammonium concentration dropped afterwards and reached a minimum of about 20 μmol·g FW^{-1} after 8 days of active photorespiration, still above WT levels. Beyond 10 days of incubation under normal CO_2 conditions, *Ljgln2-2* plants showed important chlorosis and necrosis of the leaves. For this reason, longer incubation times were not considered in this study.

A concerted repression of photorespiratory genes is triggered by photorespiratory conditions in the *Ljgln2-2* mutant

The ammonium levels in the *Ljgln2-2* mutant showed a drop after a maximum level has been attained (Fig. 1). This was suggestive of the existence of a regulation of the photorespiratory metabolism. In order to investigate this, the expression of *L. japonicus* photorespiratory genes was quantified by qRT-PCR in WT and mutant plants at the same time points considered in Fig. 1. The expression levels of most of the genes analyzed showed a sudden drop after the transfer to normal CO_2 conditions in the mutant, with a reduction in transcript levels of about 70-80% after two or three days, followed in most cases by a slow recuperation (Fig. 2). This was not the case for the WT plants, where most genes did not show any clear tendency and only three of them (*LjGln2*, *LjGlu1* and *LjGlyK1*, encoding for plastidic glutamine synthetase, ferredoxin-dependent GOGAT and a glycerate kinase isoform respectively) were repressed more than two-fold after the transfer to normal CO_2 conditions (Fig. 2, Supplemental Fig. S1). The coordinate repression and the similar trend in transcript levels observed for most photorespiratory genes in the mutant indicated that *L. japonicus* photorespiratory genes could be regulated in a common way. This is, to our knowledge, the first direct experimental evidence for a coordinate regulation of photorespiratory genes over time. For reason of space, the complete time course of transcript levels is presented in Fig. 2 for 6 of the 23 genes analyzed.

However, the common transcriptional trend of the photorespiratory genes in the mutant is also evident when considering two time points: 2 days, where transcription is often minimal, and 8 days where the transcript levels show a slight recuperation. Significant differences in transcript levels between the two genotypes at these two key time points are highlighted in bold in Table 1. The complete transcriptional time-course for all the photorespiratory genes is available online (Supplemental Fig. S1).

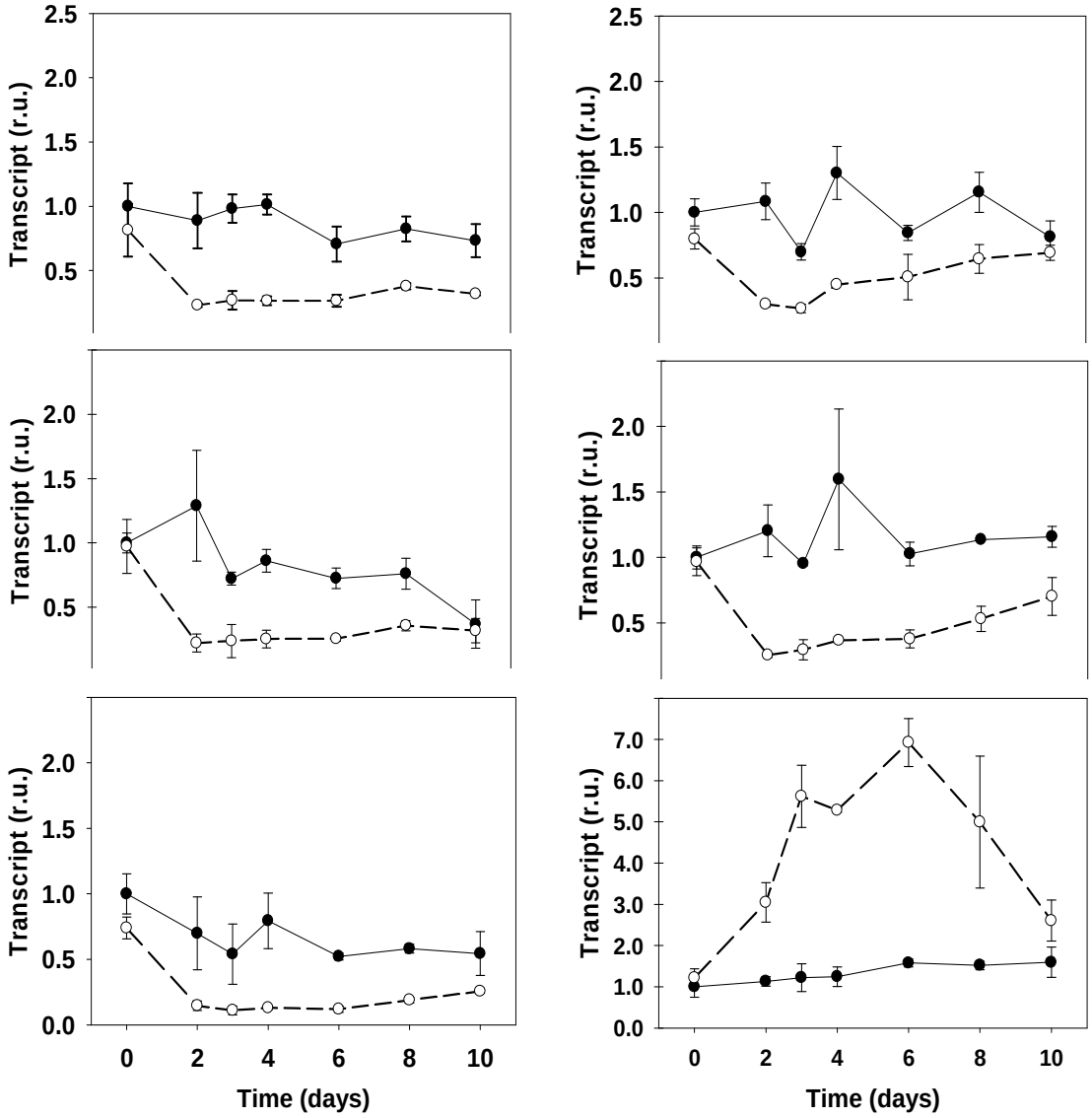

Figure 2. Expression of some photorespiratory genes in *Lotus japonicus* plants under active photorespiration. WT (black dots, solid line) and *Ljgln2-2* (white dots, dashed line) plants grown for 35 days in high CO_2 (time zero) were transferred to normal CO_2 conditions for the indicated periods of time. Leaves were harvested at the indicated time points for the quantification of different transcripts by qRT-PCR. Transcript levels are reported as relative units (r.u.). For comparative purposes, the transcript levels measured in the WT plants under high CO_2 conditions (time 0) were taken as 1. The expression profile of the following genes is presented: RUBISCO small subunit (*LjRbc_s1*, probeset chr2.TM1655.9); the H subunit of glycine decarboxylase (*LjGDCH1*, probeset Ljwgs_010991.0.1); serine:glyoxylate aminotransferase (*LjSGAT2*, probeset Ljwgs_079709.1); hydroxypyruvate reductase (*LjHPR*, probeset Ljwgs_011418.2); glycolate oxidase (*LjGO1*, probeset Ljwgs_013523.1) and the plastidic isoform of glutamine synthetase (*LjGln2*, probesets gi18266052 and TM1765.11). Data for all the measured genes are available online (Supporting Information Fig. S1). Data are the mean ± S.D. of three independent biological replicates.

	WT			*Ljgln2-2*		
Time (days)	**0**	**2**	**8**	**0**	**2**	**8**
Gene						
LjRbc_l	1.00	**1.21**	**0.96**	0.86	**0.34**	**0.43**
LjRbc_s1	1.00	**1.03**	**0.92**	1.12	**0.02**	**0.03**
LjRbc_s2	1.00	**0.89**	**0.82**	0.81	**0.23**	**0.38**
LjPglP1	**1.00**	**0.62**	**0.81**	**0.78**	**0.16**	**0.31**
LjPglP2	**1.00**	**0.69**	**1.07**	**0.80**	**0.19**	**0.40**
LjGO1	1.00	**1.13**	**1.52**	1.21	**3.05**	**5.00**
LjGO2	1.00	**1.31**	**1.86**	1.05	**0.29**	**1.04**
LjSGAT1	1.00	**1.12**	2.01	1.41	**0.65**	2.61
LjSGAT2	**1.00**	**1.08**	**1.15**	**0.80**	**0.30**	**0.65**
LjGDCH1	**1.00**	**0.70**	**0.58**	**0.74**	**0.15**	**0.19**
LjGDCH2	**1.00**	**0.88**	**0.81**	**0.76**	**0.24**	**0.34**
LjGDCP1	1.00	**1.29**	**1.55**	1.04	**0.31**	**0.96**
LjGDCP2	1.00	**1.02**	**1.38**	1.07	**0.19**	**0.63**
LjGDCT	1.00	**0.98**	**0.96**	0.80	**0.18**	**0.33**
LjSHM1	**1.00**	**0.89**	**0.85**	**0.73**	**0.19**	**0.35**
LjSHM2	1.00	0.78	**1.00**	0.66	0.59	**0.57**
LjHPR	1.00	**1.20**	**1.14**	0.97	**0.25**	**0.53**
LjGlyK1	1.00	0.44	0.31	0.81	0.36	0.61
LjGlyK2	**1.00**	**0.79**	**0.76**	**0.70**	**0.21**	**0.22**
LjGln2	1.00	**1.29**	**0.76**	0.97	**0.22**	**0.36**
LjGlu1	1.00	**0.77**	**0.76**	1.31	**0.29**	**0.38**
LjDiT1	1.00	**1.21**	**2.28**	1.45	**0.43**	**0.99**
LjDiT2.1	1.00	**0.95**	**1.53**	0.79	**0.29**	**0.36**

Table 1. Expression levels of photorespiratory genes at selected time points. The transcript levels for WT and *Ljgln2-2* mutants at time 0 (high CO_2) and after 2 and 8 days of transfer to normal CO_2 conditions are reported. For comparative purposes, the transcript levels in WT plants under high CO_2 conditions (time 0) were taken as 1. Numbers in bold mean significant difference between genotypes at one specific time point according to Student's t-test ($P < 0.05$).

Induction of *LjGO1* is associated with increased glycolate oxidase activity and H_2O_2 content in the *Ljgln2-2* mutant

The most striking exception to the common transcriptional trend showed by photorespiratory genes was *LjGO1*, one of the two genes found in the *L. japonicus* genome encoding for glycolate oxidase (GO) (Fig. 2, Table 1). While *LjGO1* was the only gene that was highly induced in the mutant, the other gene encoding for GO (*LjGO2*) was repressed in this genotype mutant like most photorespiratory genes (Table 1, Supplemental Fig. S1). This peculiar transcriptional behaviour was of particular interest since an eventual increase of GO activity may lead to high levels of H_2O_2. Production of hydrogen peroxide by GO has a strongly negative effect on plants (Maier et al., 2012) and may contribute to *Ljgln2-2* phenotype. Interestingly, recent works suggested that H_2O_2 production by GO may be increased upon re-activation of photorespiration in

Arabidopsis (Timm et al., 2012). For this reason, the levels of GO enzyme activity and the H_2O_2 content were determined in leaves of WT and mutant plants at all the time points considered (Fig. 3). GO activity was increased about 50% in the mutant after the transfer to normal air conditions, in quite good agreement with the increase in *LjGO1* transcript levels observed in the mutant (Fig. 2). Moreover, the decrease of *LjGO1* transcript levels in the mutant after the maximum attained (Fig. 2) was also paralleled by a decrease in enzyme activity. In fact, GO activity at days 2 and 3 in the mutant was significantly higher than the activity observed at day 10 for the same genotype ($P < 0.05$ according to Student's t-test, not shown in Fig. 3). H_2O_2 content in mutant leaves was slightly increased after the first days of transfer to normal CO_2 conditions and diminished after an initial maximum (Fig. 3B). Even if stastically significant, the differences in H_2O_2 content between WT and mutant are probably too modest to account for the observed differences in transcript and metabolite abundance. On the other hand, the data presented strongly suggest that the induction of *LjGO1* is responsible at least in part of the increase of GO activity in *Ljgln2-2* under normal CO_2 conditions.

In order to get further insight into the different regulation of the two *GO* genes, a co-expression study was carried out in order to identify the top 100 genes with the most similar transcriptional profile to either *LjGO1* or *LjGO2* as described in Materials and Methods.

Several of the 100 top *LjGO2* co-expressed genes were linked to photosynthetic metabolism like photorespiratory, photosynthetic and Calvin cycle genes (Supplemental Fig. S2). However, this was not the case for *LjGO1*. In contrast, 23 of the 100 *LjGO1* co-expressed genes were related to stress response, while in the case of *LjGO2* only 10 of the top 100 co-expressed genes belonged to the stress response category according to the MapMan software (Supplemental Fig. S2). Recent studies have demonstrated that glycolate oxidase is involved in stress response and pathogen resistance in plants (Taler et al., 2004; Rojas et al., 2012), probably by means of H_2O_2 production. The data presented here clearly indicate that *LjGO2* is regulated in a similar way to other photorespiratory genes while *LjGO1* may be involved in the response to stress in *L. japonicus*. If this hypothesis is true, the induction of *LjGO1* in the mutant plants may represent a generic stress response due to the metabolic unbalance observed in the mutant under active photorespiratory conditions as a result of the impairment of the C2 photorespiratory cycle.

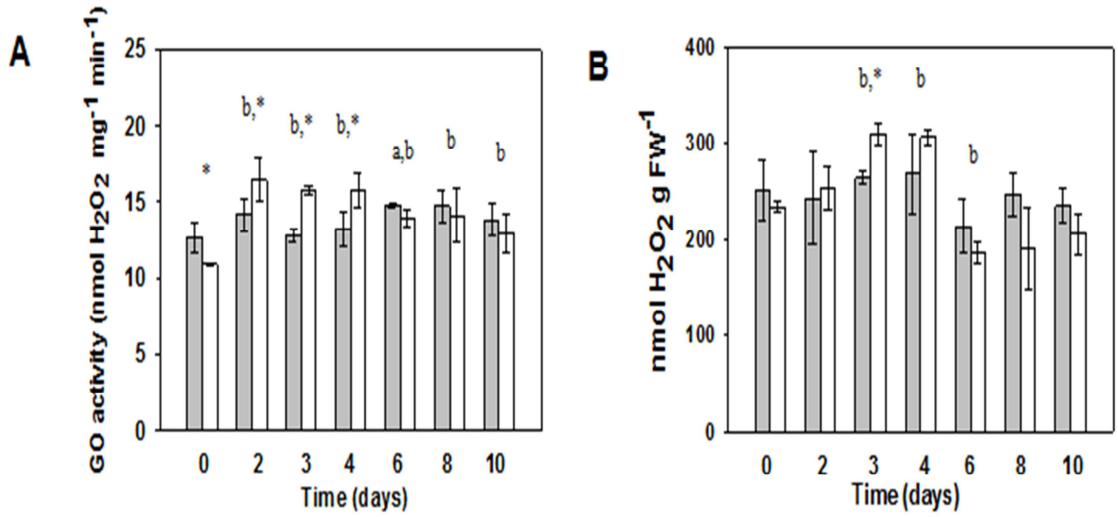

Figure 3. Glycolate oxidase activity (A) and H_2O_2 content (B) in WT and *Ljgln2-2* mutant leaves. WT (grey bars) and *Ljgln2-2* (white bars) plants grown for 35 days in high CO_2 (time zero) were transferred to normal CO_2 conditions for the indicated periods of time. Glycolate oxidase (GO) activity is expressed as nmol of H_2O_2 produced per minute and mg of total protein. H_2O_2 is expressed as nmol of H_2O_2 per mg of leaf fresh weight. Data are the mean ± S.D. of three independent biological replicates. a: significant difference between WT at this time point and WT at time zero; b: significant difference between *Ljgln2-2* at this time point and *Ljgln2-2* at time zero; *: significant difference between WT and *Ljgln2-2* at this time point. Significant differences are defined as $P < 0.05$ according to Student's t-test.

It is well known that different types of stresses share an overlapping group of genes (Fujita et al., 2006) and it is may be possible that the induction of *LjGO1* may be associated to this generic stress response. Moreover, it has to be taken into account that the data presented here were obtained at the level of whole leaf, and do not reveal the anatomical distribution of these two GO isoform. Further studies at greater anatomical resolution should be carried out in order to obtain a more detailed scenario of the transcriptional regulation of GO1 and GO2 in different cell types. It may be possible that greater changes in H_2O_2 may accompany altered expression of both GO1 and GO2 in the corresponding cell types.

Photorespiratory and photosynthetic genes show a similar transcriptional regulation

Recent analysis of Arabidopsis microarray data suggested that photorespiratory and photosynthetic genes respond in a similar way to environmental changes (Foyer et al., 2009). In order to test this hypothesis, the expression of some genes of photosystems I, II and cytochrome b6f complex was quantified by qRT-PCR in WT and *Ljgln2-2* mutant plants. A drop and recover

tendency, strikingly similar to the observed for the photorespiratory genes was observed for the transcript levels of the photosynthetic genes in the mutant (Supplemental Fig. S3). This demonstrates that photorespiratory and photosynthetic genes are regulated in a similar way in *Ljgln2-2*. In contrast,, transcriptomic analysis of the response to drought stress of *Ljgln2-2* showed a general repression of the photosynthetic genes that was not paralleled by the photorespiratory ones (Díaz et al., 2010), thus suggesting that the photosynthetic and photorespiratory pathways may have both common and independent regulatory mechanisms in *L. japonicus*. Nevertheless, care should be taken in the interpretation of transcript abundance, especially in the case of photosynthetic genes. Transcript levels of photosynthetic genes may not necessarily reflect protein abundance in the thylakoid membrane considering that some of these proteins undergo a high turnover, like the photosystem II D1 protein (Takahashi et al., 2007).

A comparative analysis of all the qRT-PCR data obtained from WT and mutant plants was carried out by hierarchical clustering. This analysis confirmed that the regulation of photorespiratory and photosynthetic genes was very similar (Fig. 4).

Only three photorespiratory genes (*LjGO1*, *LjSHM2* and *LjGlyK1*) showed an important deviation from the common trend and grouped in a separate cluster (Fig. 4, upper cluster). As mentioned before, *LjGO1* was induced exclusively in the mutant, while *LjSHM2* and *LjGlyK1* were repressed both in WT and mutant plants (Table 1, Supplemental Fig. S1). It is interesting to notice that for each of the three photorespiratory genes that clustered separately, another gene copy coding for the same enzyme activity followed the common transcriptional trend. This may suggest that the gene copy regulated in the common fashion may encode for an enzyme involved in photosynthetic/photorespiratory metabolism, while the other copy may be carrying out a different function.

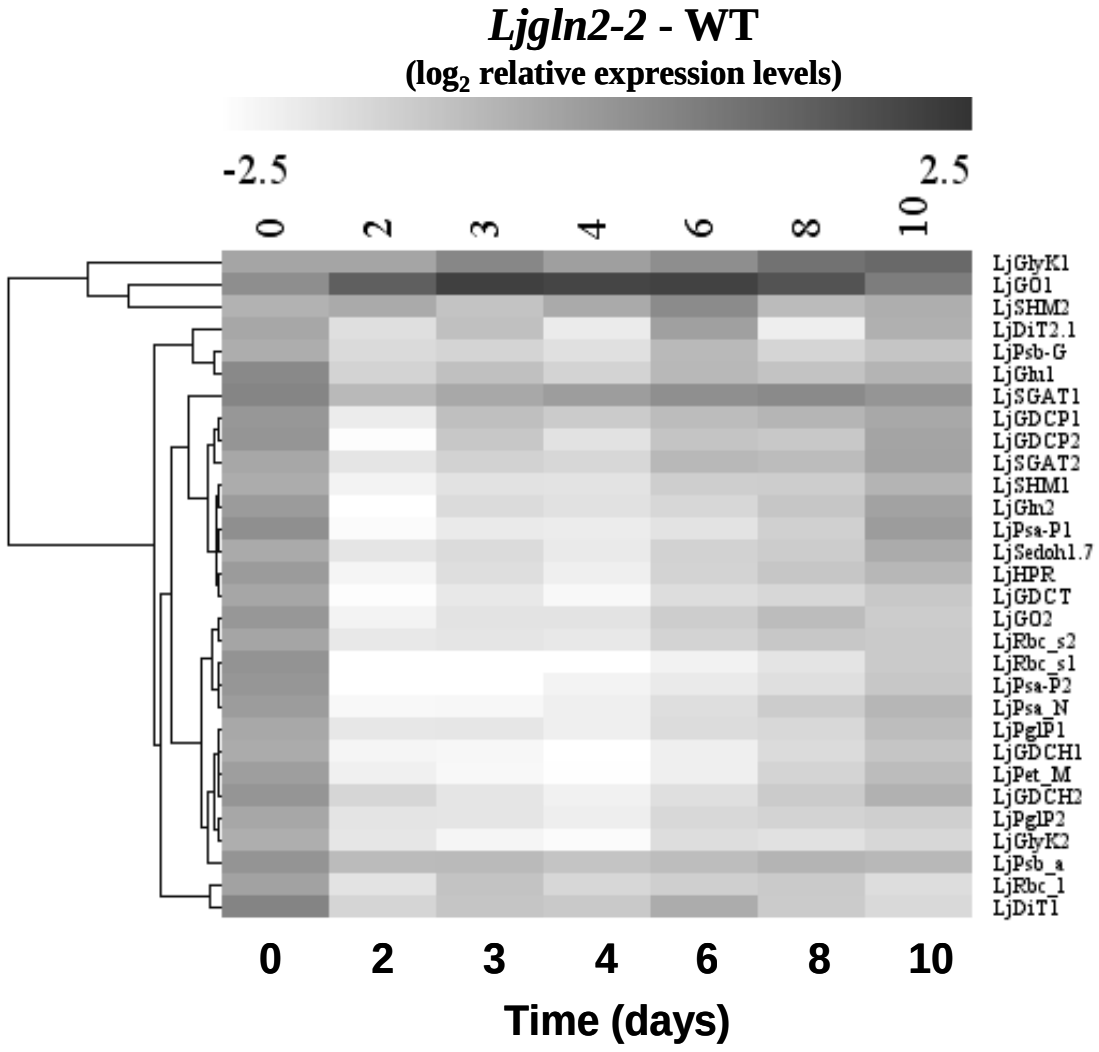

Figure 4. Hierarchical clustering analysis of qRT-PCR data for photorespiratory and photosynthetic genes. Transcript levels were determined at the indicated time periods after the transfer of WT and mutant plants from high CO_2 (time zero) to normal CO_2 conditions. Relative transcript levels of WT plants under high CO_2were taken as zero. Data are presented as the log2 of the difference of transcript levels between WT and *Ljgln2-2*. The genes considered were: glycerate kinase (*LjGlyK1* and *LjGlyK2*); glycolate oxidase (*LjGO1* and *LjGO2*); serine hydroxymethyltransferase (*LjSHM1* and *LjSHM2*); plastidic dicarboxylate transporter (*LjDiT1* and *LjDiT2.1*); photosystem II reaction centre protein G (*LjPsb_G*); ferredoxin-dependent GOGAT (*LjGlu1*); serine:glyoxylate aminotransferase (*LjSGAT1* and *LjSGAT2*); glycine decarboxylase P subunit (*LjGDCP1* and *LjGDCP2*); plastidic glutamine synthetase (*LjGln2*); photosystem I P subunit (*LjPsa_P1* and *LjPsa_P2*); sedoheptulose 1,7 bisphosphatase (*LjSedoh1.7*); hydroxypyruvate reductase (*LjHPR*); glycine decarboxylase T subunit (*LjGDCT*); rubisco small subunit (*LjRbc_s1* and *LjRbc_s2*); photosystem I reaction centre subunit N (*LjPsa_N*); phosphoglycolate phosphatase (*LjPglP1* and *LjPglP2*); glycine decarboxylase H subunit (*LjGDCH1* and *LjGDCH2*); cytochrome b6f complex subunit (*LjPet_M*); photosystem II protein D1 (*LjPsb_a*) and the gene for the RUBISCO large subunit (*LjRbc_l*).

Transfer from high CO_2 to normal air conditions produces massive transcriptomic changes in the *Ljgln2-2* mutant and minor but significant ones in the WT

In order to determine if the transfer from high CO_2 to normal CO_2 conditions may have an influence on the transcription of other genes than the involved in photosynthesis and photorespiration, a global transcriptomic study was carried out in leaves of WT and mutant plants. For this aim, the recently developed Affymetrix Lotus Genechips® (Sánchez et al., 2008; Guether et al., 2009; Högslund et al., 2009; Díaz et al., 2010) was used with three independent biological replicates for each genotype and time point. The analysis was carried out at time zero (high CO_2) and after 2 days of exposition to normal CO_2, in which the plants showed active photorespiration associated with transcriptional changes, together with almost maximum levels of ammonium, but did not show apparent symptoms of chlorosis.

Changes in gene expression between WT and *Ljgln2-2* plants were analyzed by a significance-based comparison applying a false discovery rate (FRD) < 0.05. No fold-change threshold was applied since recent transcriptomic studies in *L. japonicus* demonstrated that subtle transcriptional changes, if statistically significant, are often biologically relevant and represent robust responses to stress conditions (Sánchez et al., 2010). Statistically changed genes were visualized and functionally characterized using MapMan, PathExpress and Genebins programs (Usadel et al., 2005; Goffard and Weiller, 2007a,b). qRT-PCR was used to validate microarray data for several genes that were significantly modulated in WT or mutant plants according to the Affychips (Supplemental Fig. S4). A remarkable good agreement between the two technologies was found, with a linear regression slope of 1.22 and r^2=0.85. Since the transcriptomic differences between WT and *Ljgln2-2* grown under high CO_2 atmosphere were recently described (Diaz et al., 2010), we focused on changes induced by the transfer from high CO_2 to normal CO_2 atmosphere.

The total number of genes modulated by the 2-days shift to photorespiratory conditions was much higher in the mutant plants: 6610 Affychip probesets were changed in the *Ljgln2-2* genotype compared to 1480 of the WT. The two genotypes shared 825 modulated probesets, corresponding to about 56% and 12% of the total modulated probesets for WT and *Ljgln2-2*, respectively. On the other hand, 655 and 5785 probesets were modulated specifically in the WT or in the mutant plants, respectively (Fig. 5A). The three groups of genes defined by the Venn diagram in Fig. 5A are analysed hereafter.

Genes modulated only in WT plants

Among the genes modulated by the transfer to normal CO_2 conditions, it was interesting to analyze separately the ones that changed exclusively in the WT genotype, since they probably represent part of the normal response of the plant to active photorespiration that is directly or indirectly dependent on plastidic GS_2 activity. A first global functional analysis of this small subset of 655 probesets was carried out using Pathexpress (Goffard and Weller, 2007a), a program that identifies the most relevant metabolic pathways associated with a subset of genes. The analysis carried out however did not discover any metabolic pathway that was significantly over-represented. For this reason, a further analysis of this group of genes was carried out using Genebins (Goffard and Weller, 2007b). This tool assigns genes to hierarchical categories (BINs) based on the ontology provided by the KEGG database, thus finding not only metabolic pathways but also cellular functions that are significantly up- or down-regulated in a microarray experiment. Genebins found that the gene families coding for three of the four core histones (H2B, H3 and H4) were statistically over-represented (Supplemental Fig. S5). Analysis of the corresponding 22 probesets indicated that the transcription of these histone genes was repressed roughly about two-fold in WT plants by the transfer to normal CO_2 conditions (Supplemental Table S1). This was curious since the transcription of the genes encoding for histone proteins is modulated mainly by the entry of the cells in S phase, rather than by external conditions. This general repression of the histone genes may indicate a reduced cellular division when WT plants are shifted from high CO_2 to normal CO_2 atmosphere. Several marker genes involved in cell division and in the control of the cell cycle were also repressed, supporting this hypothesis (Supplemental Table S1). qRT-PCR analysis of selected genes encoding for different histone proteins and cyclins confirmed the down-regulation depicted by the microarray study (Supplemental Table S2).

The transcription of photorespiratory genes was almost unaffected in the WT by the transfer to normal CO_2 conditions, confirming the qRT-PCR data. This probably indicates that the WT plants do not need to modulate the expression of photorespiratory genes in order to cope with the transfer to photorespiratory active conditions. The full list of genes from this subset, together with the genes modulated in both genotypes and exclusively in the mutant as well as their MapMan overview of general metabolism are available online (Supplemental Table S1, Supplemental Fig. S6).

Genes modulated both in WT and *Ljgln2-2* mutant plants

In order to study the core/common response of *L. japonicus* to the change from high CO_2 to normal CO_2 atmosphere that is not dependent on plastidic GS_2, we analyzed the group of 825 probesets that were modulated in both genotypes. The analysis of the fold change of these sequences revealed that about 90% of them (739 out of 825) changed in the same direction in the two plant genotypes (Fig. 5B). Interestingly, linear regression of the $\log_2$ of the fold change for these genes in WT and mutant plants gave an angular coefficient value of 1.39 indicating that on average, these genes were elicited more than two-fold ($2^{1.39}$ = 2.62) in *Ljgln2-2* compared to the WT. Díaz et al. (2010) demonstrated that most of the genes that changed both in WT and *Ljgln2-2* in response to drought were also more modulated in the mutant. This suggested proportionality between the extent of the gene modulation and the level of stress perceived/received by the *Ljgln2-2* mutant plants (Diaz et al. 2010).

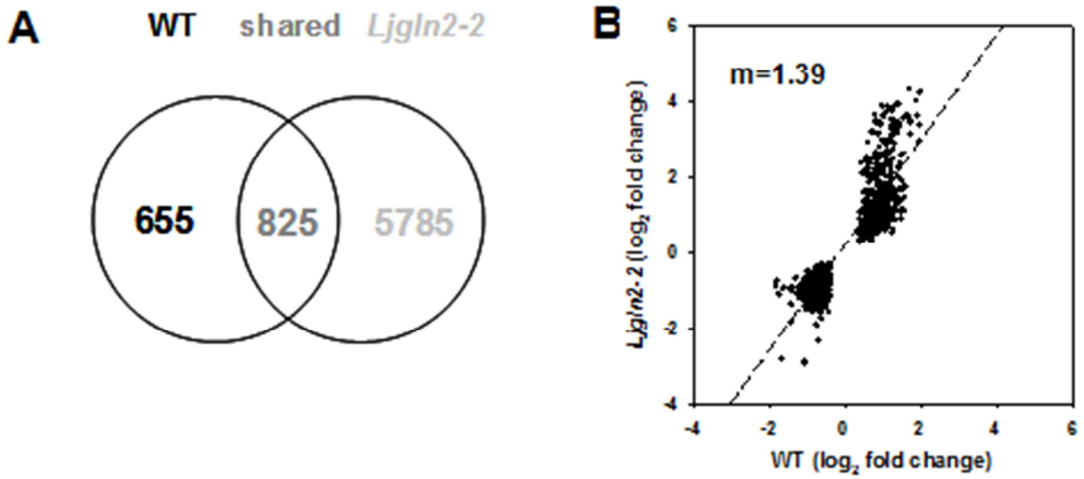

Figure 5. Genes elicited by the transfer from high CO_2 to normal CO_2 conditions in WT and *Ljgln2-2* mutant plants. (A) Venn diagram representing the number of genes elicited by the transfer from high to normal CO_2 conditions in WT and *Ljgln2-2* mutant plants. Significantly modulated genes were identified by comparing the $\log_2$ of the mean of expression levels at 2 days and time zero and applying a false discovery rate (FDR) of < 0.05. The expression levels at time zero in the WT or in the mutant were used as a control for the WT and the mutant respectively. (B) Comparison of the $\log_2$ of fold change value for the genes significantly elicited in both genotypes that changed in the same direction. A linear regression analysis was carried out (r^2 = 0.80, m = 1.39).

Analysis of the data using Pathexpress gave three significantly more represented pathways: starch and sucrose metabolism, flavonoids biosynthesis and stilbene/lignin/coumarin biosynthesis (Supplemental Fig. S7). In the first case, down-regulation of the genes for both starch and sucrose degradation was

observed (Supplemental Fig. S8), suggesting that the drop in CO_2 concentration may be influencing carbohydrate metabolism in both genotypes. This is consistent with a decreased starch production in conditions of lower CO_2 availability and increased photorespiration. According with this hypothesis, starch and sucrose levels in the nodules of WT and *Ljgln2-2* were reduced by the transfer from high CO_2 to normal CO_2 conditions (García-Calderón et al., 2012). Several genes for both flavonoids and stilbene/lignin/coumarin biosynthesis were induced upon transfer to normal CO_2 conditions, including genes encoding for key enzymes of these routes like chalcone synthase and isoflavone reductase (Supplemental Table S3). Flavonoids are a group of secondary metabolites belonging to the family of phenylpropanoids with different functions in plants, including protection from pathogens or ultraviolet light, and they can also act as scavengers of reactive oxygen species. Previous transcriptomic experiments carried out with salt-stressed WT *Lotus japonicus* plants (Sánchez et al., 2008) and drought-stressed WT and *Ljgln2-2* mutant plants (Díaz et al., 2010) also reported the induction of genes for flavonoids biosynthesis, indicating that this is a common response of *L. japonicus* to different abiotic stresses. The last more represented pathway identified, stilbene/lignin/coumarin metabolism, is part of the larger phenylpropanoid pathway. The modulated genes identified within this group were essentially the same than the ones for flavonoid biosynthesis, as they represent common steps in the synthesis of different classes of phenylpropanoids. The repression of some key genes in starch and sucrose degradation and the induction of isoforms of chalcone synthase and isoflavone reductase in both genotypes were confirmend by qRT-PCR analysis (Supplemental Table S2).

Analysis of this group of genes with Genebins gave 33 over-represented pathways and cellular functions, which can be classified mainly in three groups: carbohydrate metabolism, secondary metabolism and redox-related genes (Supplemental Fig. S5). While the physiological relevance of altered carbohydrate and secondary metabolism has been discussed previously, the modulation of redox-related genes observed also indicates that the atmospheric CO_2 concentration is able to affect the cellular redox state. Recent studies with Arabidopsis confirms this hypothesis (Queval et al., 2012 and references therein). Among the group of genes recognized by Genebins the pathways for starch and sucrose metabolism, flavonoids biosynthesis and stilbene/lignin/coumarin biosynthesis were defined as over-represented, confirming the results obtained with Pathexpress. A list of the genes belonging to these three pathways according to either Genebins or Pathexpress is available online as Supplemental Table S3 for comparative purposes.

Genes modulated only in *Ljgln2-2* mutant plants

The majority of the modulated probesets (almost 6000) corresponded to genes that changed specifically in *Ljgln2-2* mutant plants by the transfer from high CO_2 to normal CO_2 conditions. Since this group of genes is elicited specifically in the absence of plastidic GS_2, it can be inferred that they are truly responding to the presence of an impaired photorespiratory cycle and/or to the high levels of ammonium accumulated by the mutant. Pathexpress analysis identified two significantly more represented pathways: porphyrin/chlorophyll metabolism and carbon fixation (essentially the Calvin cycle) (Supplemental Fig. S7). The MapMan overview of photosynthesis-related genes showed that most photosynthetic genes, as well as several photorespiratory genes, were repressed exclusively in the mutant (Supplemental Fig. S9), confirming the data obtained by qRT-PCR. Moreover, several genes of the Calvin cycle and of the biosynthesis of photosynthetic pigments were repressed, indicating that there is a general shutdown of photosynthetic metabolism in the mutant. qRT-PCR analysis confirmed that genes encoding for enzymes of the Calvin cycle like sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase and key genes in porphirin/chloroplyll metabolism like glutamyl-tRNA reductase and magnesium chelatase were repressed in the mutant genotype (Supplemental Table S2). It is worth to notice that a mutation in a gene of nitrogen metabolism like *LjGln2* affects the transcription of the genes of Calvin cycle, reflecting the tight interaction between C and N metabolism.

Genebins analysis of the genes specifically elicited in *Ljgln2-2* detected more than 120 over-represented gene families, spanning the majority of the primary and secondary metabolism, including for example carbon and nitrogen metabolism, biosynthesis of chlorophyll, amino acids metabolism, glycolysis, the Krebs cycle and starch/sucrose metabolism, among others (Supplemental Fig. S5). This indicates that impairment of the photorespiratory cycle and/or the subsequent accumulation of ammonium have a global effect on the metabolism of the leaf, revealing the crucial importance of the reassimilation of photorespiratory NH_4^+ and the multiple metabolic interconnections of it. Once again, Genebins analysis confirmed that the two pathways recognized by Pathexpress, porphyrin/chlorophyll metabolism and carbon fixation were over-represented (Supplemental Fig. S5, Supplemental Table S3).

Many genes associated with photorespiration such as those for transporters involved in substrate flow between the chloroplast, the mitochondrion and the

peroxisome have not been, with counted exceptions, characterised (Bauwe et al., 2010; Peterhansel et al., 2010; Weber and von Caemmer, 2010; Eisenhut et al., 2013; Pick et al., 2013). Interestingly, we found 287 transporter gene sequences within the group of genes modulated exclusively in the mutant, several of them with unknown function. Future experiments should be designed in order to characterise these transporter genes and, particularly, to determine if any of them may be involved in the transport of photorespiratory metabolites.

Metabolite profiling analysis

The transcriptomic analysis carried out for the *Ljgln2-2* mutant was complemented with further metabolic analysis using gas chromatography-mass spectrometry technology (GC/EI-TOF-MS). Changes in the levels of soluble metabolites between WT and mutant plants were examined at the different time points considered in Figs. 1, 2, 3 and 4. For a targeted analysis of metabolites, we compiled a total of 202 mass spectral tags (MSTs, i.e. manually recognized analytes, Desbrosses et al., 2005), including known and yet unknown compounds (Supplemental Table S4). Of the latter group, 31 MSTs represented unknown metabolites which were so far found only in *Lotus* species (indicated by a D code, Sánchez et al., 2011). The metabolite profiles were statistically analyzed with a supervised two-factorial ANOVA at a stringent threshold ($P < 0.0001$), using the factors "genotype" (*Ljgln2-2* and WT) and "time of exposure to normal CO_2" (0, 2, 3, 4, 6, 8 and 10 days after transfer from high CO_2 to normal CO_2 atmosphere). A total of 100 MSTs were found to be significantly changed, most of them due to the genotype factor (94 MSTs), thus demonstrating a profound metabolic effect of the *Ljgln2-2* mutation upon exposure to photorespiratory conditions (Supplemental Table S4). 5 different patterns of behaviour were recognized as global metabolomic responses of the statistically changed MSTs (Fig. 6):

(a) Metabolites that increased in the mutant upon transfer to normal CO_2 conditions with no change in the WT (40 MSTs); in many cases their content was low under a high CO_2 atmosphere. This group included several amino acids including Gly, Leu, Ile, Val, Trp, Phe and organic acids such as malate, citrate, succinate and 2-oxoglutarate.

(b) Metabolites that increased in the mutant upon transfer to normal CO_2 conditions, but decreased in the WT (8 MSTs), including mannose, glucose, glucose-6-P and glutamine.

(c) Metabolites that showed a higher content in the mutant background under high CO_2 conditions, which sometimes increased upon transfer to normal CO_2 conditions only in the mutant (23 MSTs). These included many unknown compounds, and particular organic acids such as saccaric acid, gluconic acid, glucaric acid-1,4-lactone, 2-isopropyl-malic acid and 2-piperidine-carboxylic acid.

(d and e) Metabolites that increased/decreased in WT or mutant but showed lower content in the mutant background under high CO_2 conditions (group d, 9 MSTs), or behave sometimes transiently or to a higher level in the mutant when the content at day 0 was similar between both genotypes (group e, 14 MSTs). These groups included metabolites such as proline, trehalose, putrescine, *myo*-inositol, and also key metabolites linking nitrogen and carbon metabolism like aspartate and glutamate.

Finally, four metabolites (Uric acid (4TMS), A182009, A180002 and A281001) were ruled out from our statistical analysis because their levels were below the detection limit in most WT samples, but in *Ljgln2-2* they displayed a high peak at days 3-4 after transfer to normal CO_2 atmosphere (Supplemental Table S4).

The first two groups suggest the existence of metabolic pathways which were secondarily elicited as a result of the metabolic toxicity due to the lack of a complete photorespiratory cycle, or in which the plastidic GS_2 is directly required for physiological acclimation to: (i) the changes induced by active photorespiration, (ii) the detoxification of ammonium, or (iii) changes in carbon level due to the transition from high CO_2 atmosphere to normal CO_2. The increase of various amino acids (Leu, Val, Ile, Trp, Phe, Lys, Gly) found exclusively in the mutant suggests that nitrogen metabolism was not a limiting factor for the mutant fitness but rather a means to ammonium detoxification. In line with this idea, the levels of these amino acids paralleled the peak of ammonium at day 3. Curiously, glutamine showed a distinctive pattern as it remained increased until day 10 in the mutant (Fig. 6, group b). This demonstrates that the *Ljgln2-2* mutant is able to produce high levels of glutamine, even in the absence of plastidic GS_2, which is the most abundant GS isoform in *L. japonicus* leaves (Orea et al., 2002; Betti et al., 2006).

Cytosolic GS_1 should be then responsible of the accumulation of glutamine in the mutant. The fact that glutamine levels in the mutant were high even after the drop in ammonium levels may also suggest that the cytosolic GS_1

isoform could be detoxifying the photorespiratory ammonium and contribute partially to the reduction of NH_4^+ levels after day 3

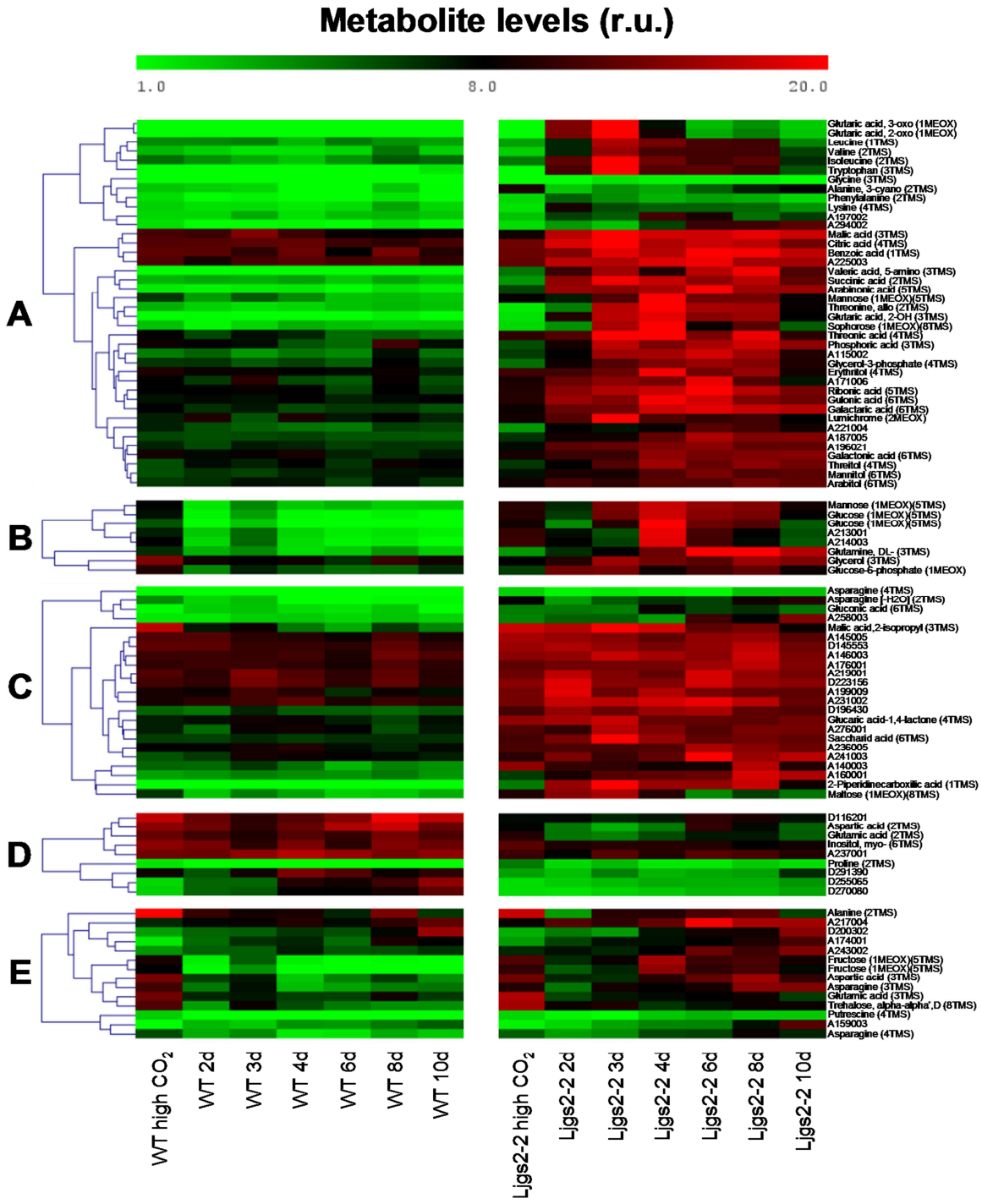

Figure 6. Metabolite profiling of WT and *Ljgln2-2* mutant plants. The metabolomic profiles of WT and *Ljgln2-2* mutant plants were statistically analyzed using a supervised two-factorial ANOVA, with "genotype" and "time of exposure to normal CO_2" (0, 2, 3, 4, 6, 8 and 10 days after transfer from high to normal CO_2 atmosphere) as factors at a stringent threshold ($P < 0.0001$). 5 different groups were defined by clustering tools. Data represent the direct normalized responses of metabolite pool measures. The color bar represents this relative unit (r.u.). Further details in Materials and Methods.

In accordance with this hypothesis, two probesets corresponding to cytosolic GS_1 (Ljwgs_088254.1 and Ljwgs_015806.2) were induced about two-fold exclusively in the mutant. A search in the gene databases of *L. japonicus* revealed the existence of at least three different genes encoding for cytosolic GS_1 in this model legume (not shown). The two probesets previously mentioned corresponded to the same *LjGln1* gene encoding for a cytosolic GS isoform located on chromosome 6 that will be called from now on *LjGln1.2*. qRT-PCR analysis confirmed that this gene was induced about two-fold in the mutant under active photorespiratory conditions (Fig. 7). The increase in *LjGln1.2* transcript was also paralleled by an increase in GS enzyme activity in *Ljgln2-2* mutant plants, which was not observed in WT plants (Fig. 7). This induction of a cytosolic GS_1 isoform may explain the increase of the glutamine pool observed in the mutant. Moreover, the increased GS_1 activity may also be involved in the reduction of the ammonium levels after the maximum attained at day 3 (Fig. 1)

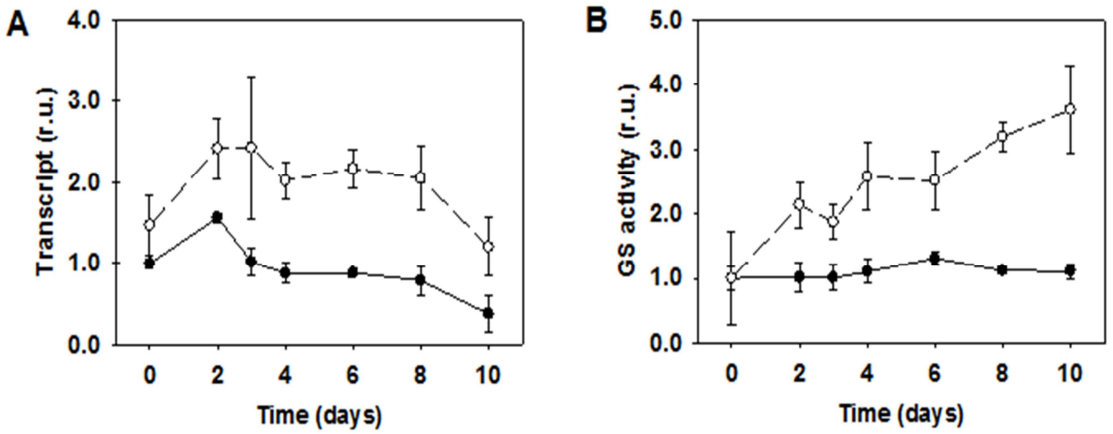

Figure 7. *LjGln1.2* cytosolic GS transcript levels (A) and total GS activity (B) in WT and *Ljgln2-2* mutant plants. (A) The relative expression levels of the *LjGln1.2* gene encoding for a cytosolic GS isoform were determined by qRT-PCR in leaves of WT (black dots, solid line) and *Ljgln2-2* (white dots, dashed line). Plants were grown under high CO_2 for 35 days and transferred for the indicated periods of time to normal CO_2 atmosphere. Transcript levels are reported as relative units (r.u.). For comparative purposes, the transcript levels measured in the WT plants before the transfer to normal CO_2 conditions (time 0) were taken as 1. (B) The GS specific activity was determined in crude extracts from leaves of WT (black dots, solid line) and *Ljgln2-2* (white dots, dashed line) at the same time points as in (A). Total GS activity is reported as relative units (r.u.). For comparative purposes, the specific GS activity at time zero in WT or in mutant leaves was taken as 1 for WT and mutant respectively. Total GS activity at time zero was 591 and 85 mU/mg protein in WT and mutant leaves respectively. Data are the mean ± S.D. of three independent biological replicates.

Group c, composed mainly of unknown metabolites, suggests the existence of metabolic pathways in which plastidic GS_2 is constitutively required for particular metabolic processes, and may, in some cases, be involved in the

acclimation to changes induced by the transfer from high CO_2 to normal CO_2 conditions.

The two last groups of metabolites (groups d and e) suggest the existence of general metabolic pathways required for the acclimation to the changes induced by the transition from high CO_2 to normal CO_2 atmosphere. Interestingly, in addition to several unknown compounds, various important nitrogen metabolites clustered in these two groups. Aspartate levels in the mutant were almost half of the WT under control conditions, while asparagine was only slightly lower in the mutant in the same conditions. This could indicate that in the mutant under non-photorespiratory conditions Asp and Asn may be used more extensively as amino donors. This is possibly related to the fact that asparagine constitutes most (86%) of the nitrogen translocated in *L. japonicus* plants (Credali et al., 2011). The decrease of glutamate in the mutant under normal CO_2 conditions is quite surprising considering that the levels of this amino acid are normally quite stable in plants (Forde and Lea, 2007), and could be explained by the great accumulation of several amino acids (Fig. 6, group a) that may drain the glutamate pool. Proline levels decreased in both genotypes as a consequence to the shift to normal CO_2 conditions. Despite of this common trend, proline levels were higher in the mutant than in the WT at all the experimental time points considered. This is surprising since it was previously described that the *Ljgln2-2* mutant accumulated lower amounts of proline than the WT under drought stress conditions (Díaz et al., 2010). Plastidic GS_2 is thus important in *L. japonicus* proline production only under particular stress conditions such as drought, but not under the stress conditions caused by impaired photorespiration. Trehalose levels also decreased in both genotypes after shift to normal CO_2 conditions. Both proline and trehaolse are molecules that accumulate in response to different kind of stresses (Sánchez et al., 2011). The similar trend shown by these two compounds indicates that the general responses against environmental changes were conserved between WT and mutant plants and that proline levels may differ in the two genotypes even in absence of osmotic stress. While most of the metabolites analyzed belonged to different pathways, four organic acids that are also intermediates of the Krebs cycle where significantly altered in the mutant indicating a relationship between plastidic GS_2 and central carbon metabolism. Since the time-course of these four metabolites was of particular interest, their time course in both genotypes is shown more in detail in Fig. 8.

Data for all the other metabolites can be found both in Fig. 6 and in Supplemental Table S4. The accumulation of NH_4^+, as well as the increase in ATP/ADP ratio and in NADH and NADPH levels in photorespiratory mutants is

known to inhibit the Krebs cycle at the level of pyruvate dehydrogenase and isocitrate dehydrogenase (Bykova et al., 2005; Bauwe et al., 2010). qRT-PCR analysis of genes encoding for components of the pyruvate dehydrogenase multienzyme complex (pyruvate dehydrogenase and dihydrolipoamide-S-acetyltransferase) and for the mitochondrial NAD-dependent isocitrate dehydrogenase (IDH) showed a general transcriptional repression over time, with the only exception of a very slight induction of IDH in the mutant at 2 days (Supplemental Table S2). Taking this into consideration, it seems unlikely that the accumulation of Krebs cycle intermediates may be related to transcriptional regulation of the genes involved in this pathway.

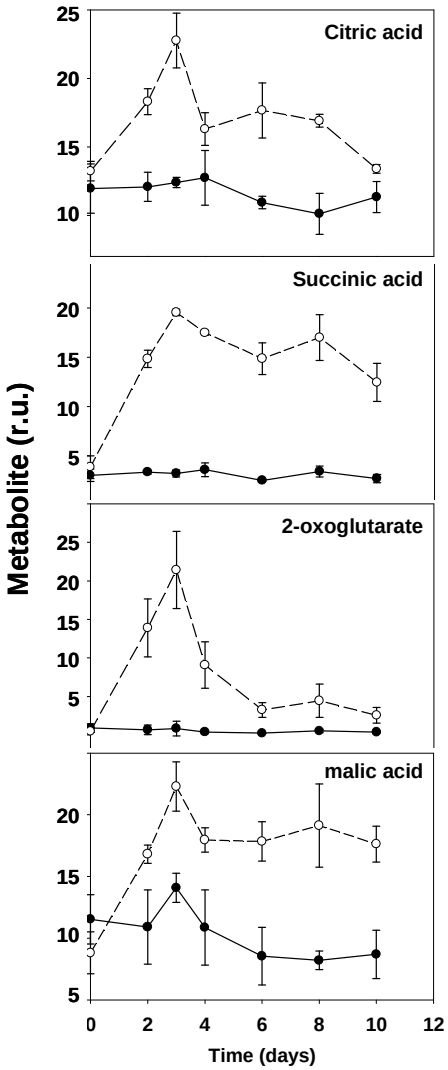

Figure 8. Time-course of some organic acids in WT and mutant plants. The relative amount of some organic acids that were defined as significantly different between WT and mutant by the statistical analysis of Fig. 6 is reported at different times after the transfer of the plants to normal CO_2 conditions. WT: black dots, solid line, *Ljgln2-2* mutant: white dots, dashed line. Metabolite levels are reported as relative units (r.u.).

On the other hand, the lack of a functional GS_2/GOGAT cycle in the chloroplast of *Ljgln2-2* may be responsible of the increase in the levels of 2-oxoglutarate, that enters the Krebs cycle and may force a higher flux of metabolites through this route. On the other hand, 2-oxoglutarate is not only a key intermediary in the Krebs cycle but also the end product of lysine degradation (Araújo et al., 2010). The accumulation of this compound may also explain the increment of many metabolites known to be upstream of this catabolic pathway (2-hydroxy-glutarate, 2-piperidine-carboxylic acid and lysine, Araújo et al., 2010). Accumulated glutaric, 2-hydroxy-glutaric and 5-amino valeric acid acids are also intermediates of lipid beta-oxidation and/or lysine and proline degradation pathways, suggesting that other catabolisms may be initiated. Supporting this hypothesis, the accumulation of branched chain amino acids together with the accumulation of Krebs cycle intermediates in Arabidopsis indicates that alternative pathways of respiration based on protein and fatty acids degradation are induced (Timm et al., 2012). Interestingly, rice mutants lacking a cytosolic GS_1 isoform showed a decrease in TCA cycle intermediates in the leaves, as well as a decrease of the aspartate family amino acids when compared to the WT (Kusano et al., 2011). While differences between the metabolite profiles of cytosolic and plastidic GS mutants are to be expected, both the data from Kusano et al. (2011) and from this study point out the central role of GS_2 in coordinating C and N metabolism in plants.

CONCLUSION

In summary, in this paper it has been demonstrated that the suppression of the key reaction of photorespiratory NH_4^+ reassimilation, catalysed by plastidic GS_2, results in several changes both at transcriptomic and metabolic level. The study of the *Ljgln2-2* mutants under photorespiratory conditions gave several novel insights on the regulation of photorespiration, photosynthesis and central metabolism in *L. japonicus*. On one hand, a coordinate repression of the photorespiratory genes was observed as a result of transfer from high CO_2 to normal CO_2 conditions exclusively in the mutant, possibly in order to avoid a further accumulation of ammonium. Several photosynthetic genes were also repressed in the mutant with a similar trend over time, indicating a common transcriptional regulation of photorespiratory and photosynthetic genes under these conditions. These transcriptional changes were part of a vast modulation of the transcriptome, especially in the mutant. The transcriptomic response was indicative of a regulation of several aspect of both primary and secondary metabolism. This was the result of impaired photorespiration during the shift

from high CO_2 to normal CO_2 conditions (modulation of photorespiratory and photosynthesis genes in the mutant), lower carbon availability (starch and sucrose metabolism, modulated in both genotypes) and higher levels of cellular stress (flavonoids and phenylpropanoids metabolism in the mutant). The levels of several metabolites were also altered reflecting transcriptomic changes, and this included several amino acids and organic acids. An increase in glutamine levels was detected in the mutant, which was paralleled by induction of cytosolic GS1 gene transcription and enzyme activity. The global panoramic of the transcripts and metabolites that changed in *L. japonicus* plants during the transfer from photorespiration-suppressed to photorespiration-active conditions clearly demonstrated how the photorespiratory cycle, besides its obliged intertwining with photosynthesis, is linked to several other cellular metabolisms, including central carbon metabolism, amino acid metabolism and secondary metabolism.

MATERIALS AND METHODS

Growth conditions and harvesting of plant material

Lotus japonicus (Regel) Larsen cv. Gifu was initially obtained from Professor Jens Stougaard (Aarhus University, Aarhus, Denmark) and then self-propagated at the University of Seville. The *Ljgln2-2* mutant, which lacks of plastidic GS_2 protein and activity, was isolated from photorespiratory mutant screening carried out using ethyl methanesulfonate as described previously (Orea et al., 2002). The mutant progeny of two consecutive backcrosses into the WT background were used. WT and mutant seeds were scarified and surface-sterilized, then germinated in 1% agar in Petri dishes and transferred to pots using vermiculite as solid support. Five seedlings were planted in each pot and grown during 35 d in a growth chamber under 16 h : 8 h day : night, 20 : 18°C, with a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1} and a constant humidity of 70%. CO_2 was automatically injected to a final concentration of 0.7% (v/v) to allow for normal growth of the *Ljgln2-2* mutant in a photorespiration-suppressed atmosphere. Plants were watered with “Hornum” nutrient solution, containing 5 mM NH_4NO_3 and 3 mM KNO_3 (Handberg and Stougaard, 1992). After 35 days of growth under high CO_2 atmosphere, leaf tissue was harvested for each plant genotype, constituting the time zero point (photorespiration suppressed conditions). The plants were then transferred to normal air (0.04% CO_2 v/v), that is active photorespiration conditions. Each plant genotype was sampled at different time points (Supplemental Fig. S9). Every harvest involved at least three independent biological replicates for each

genotype/time point from the same experiment. A biological replicate consisted of tissue pooled from five plants grown in the same pot.

RNA extraction and qRT-PCR

Leaf material was flash frozen in liquid nitrogen, homogenized with a mortar and pestle and kept at −80°C until use. Three independent biological replicates were used for the transcriptomic and quantitative real-time RT-PCR (qRT-PCR) analyses as well as for metabolite profiling analysis. Total RNA was isolated using the hot borate method (Sánchez et al., 2008). The integrity and concentration of the RNA preparations were checked using an Experion bioanalyzer (Bio-Rad) with RNA StdSens chips and a Nano-Drop ND-1000 (Nano-Drop Technologies), respectively.

For qRT-PCR analysis, total RNA was treated with the TURBO DNA-free DNAse (Ambion). Reverse transcription was carried out using SuperScript III reverse transcriptase (Invitrogen), OligodT and RNAsin RNAse inhibitor (Ambion). DNA contamination and RNA integrity were checked by carrying out qRT-PCR reactions with oligonucleotides that amplified an intron in the *LjHAR1* gene and the 3' and 5' ends of the *L. japonicus* glyceraldehyde-3-phosphate dehydrogenase respectively. qRT-PCR reactions were carried out in 10 µL in a Lightcycler 480 thermal cycler (Roche) using a SensiFAST SYBR No-ROX Kit (Bioline). Expression data were normalized using the geometric mean of four housekeeping genes: *LjGPI*-anchored protein (chr3.CM0047.42), *LjPp2A* (chr2.CM0310.22), *LjUbc10* (chr1.TM0487.4) and *LjUbq* (chr5.CM0956.27) that were selected amongst the most stably expressed genes in plants (Czechowski et al., 2005). A list of all the oligonucleotides used is provided in Supplemental Table S5.

DNA chip hybridisation and data analysis

For the transcriptomics experiments, RNA samples were labelled using the One-Cycle Target Labelling Kit (Affymetrix), hybridized to the Affymetrix GeneChip® Lotus1a520343 and scanned according to the manufacturer's instructions. MIAME compliant data were deposited at Array Express (http://www.ebi.ac.uk/arrayexpr1ess) as E-MEXP-3603. The differentially expressed genes were visualised using the MapMan program (Usadel et al., 2005) and analysed according to the corresponding metabolic pathways or functional categories using Pathexpress and Genebins (Goffard and Weiller, 2007a,b). The default thresholds of $P < 0.1$ with FDR and $P < 0.05$ with

Bonferroni correction were used for Pathexpress and Genebins respectively. Co-expression studies were carried out using the profile matching tool at the "Lotus Transcript Profiling Resource" website (http://cgi-www.cs.au.dk/cgi-compbio/Niels/index.cgi; Høgslund et al., 2009). Probesets Ljwgs_013523.1_at and Ljwgs_038509.1_at were used to search the database for genes with similar expression profiles to *LjGO1* and *LjGO2* respectively. A Pearson distance cut-off value of < 0.2 was applied in order to consider only highly positively co-expressed genes. Gene sequence searches were carried out at the Kazusa database (http://www.kazusa.or.jp/lotus/) and at the TIGR gene index (http://plantta.jcvi.org/index.shtml).

Determination of ammonium, H_2O_2 and enzyme activities

Ammonia determination was carried out according to the method of Solorzano (1969) with some modifications as described by Orea et al. (2002).

H_2O_2 determination was carried out according to Rao et al. (2000) with some modifications. Frozen leaves (0.1 g) were ground to a powder with mortar and pestle under liquid nitrogen and homogenized in 0.5 mL of 0.2 N $HCLO_4$. The homogenate was held on ice for 5 min and centrifuged at 10,000 g for 15 min and 4ºC. 0.2 mL of the supernatant were neutralized to pH 7.7 with 1 mL of NH_4OH 0.2 N pH 9.4 and centrifuged for 3 min at 3,000 g. The coloured compound in the extract were removed by applying 0.5 mL of the extract to a pre-packed 2 mL AG 1-X8 Poly-Prep column (Bio-Rad) and the soluble metabolites eluted with 3 mL of Milli-Q water. H_2O_2 was quantified using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Invitrogen). 200 µL of soluble metabolites were incubated for 30 min at 30ºC with 200 µL of Amplex reaction mix. The fluorescence at 583 nm was determined with a Hitachi F-2500 fluorescence spectrophotometer and H_2O_2 quantified by comparison with a standard fluoresence curve constructed with 3% (w/w) H_2O_2 provided with the kit. Glycolate oxidase activity was determined according to Hall et al. (1985) with some modifications. Samples (about 0.1 g) of frozen leaves that were ground to a powder with mortar and pestle under liquid nitrogen were homogenized in 5 mL/g FW of 100 mM phosphate buffer pH 8.0 at 4ºC using a pellet homogenizer. The homogenate was centrifuged for 15 min at 15,000 g and 4ºC and the supernatant was used for the enzyme activity assay. The reaction mix contained, in a final volume of 1 mL: 100 mM potassium phosphate buffer pH 8.0; 5 mM glycolate; 1 mM 4-amino-antipyrine; 0.1 mM flavin mononucleotide; 2 mM phenol; 5 U of horseradish peroxidase and 50 µL of enzyme extract. The reaction was started by addition of enzyme extract and incubated for 10 min at

30ºC and water substituted glycolate in the blank. The H_2O_2 produced by glycolate oxidase was quantified spectrophotometrically at 520 nm. Under these conditions the assay was linear up to 20 min at 30ºC and 100 µL of *L. japonicus* leaf crude extract.

GS activity was determined using the biosynthetic enzyme assay as described by Márquez (2005). Samples of frozen leaves were homogenized in 5 mL/g FW of extraction buffer. 4 µl of a 1:10 dilution of the crude extract were added to the reaction mix and incubated for 15 min at 37ºC; the phosphate released by ATP hydrolysis was quantified using the malachite green method as described by Márquez (2005).

Metabolite profiling analysis

For metabolite profiling, 60 mg of frozen plant tissue were extracted with methanol/chloroform, and the polar fraction was prepared by liquid partitioning into water and derivatized (Desbrosses et al., 2005). Gas chromatography coupled to electron impact ionization-time of flight-mass spectrometry (GC/EI-TOF-MS) was performed using an Agilent 6890N24 gas chromatograph (Agilent) with splitless injection mounted to a Pegasus III time-of-flight mass spectrometer (LECO) (Wagner et al., 2003). Metabolite-features were quantified after mass spectral deconvolution (ChromaTOF software 1.00, Pegasus driver 1.61, LECO), and their chemical identification was manually assessed using the NIST05 software (http://www.nist.gov/srd/mslist.html) and the mass spectral and retention time index collection of the Golm Metabolome Database (Kopka et al., 2005). Metabolite profiles were analyzed with the TagFinder software (Luedemann et al., 2008) and filtered for those metabolic-features represented by 3 or more inter-correlated mass fragments within each independent experiment. The validity of this analytical approach to quantify metabolites in plant tissues has been demonstrated (Allwood et al., 2009). The resulting data were normalized to internal standard (a mixture of ribitol, 2,3,3,3-d_4-DL-alanine and D(−)-isoascorbic acid) and fresh weight. All the mass spectra of the recognized MSTs can be obtained from http://gmd.mpimp-golm.mpg.de/ using the corresponding ID number.

Statistical data analysis

Microarray data were normalized with the RMA algorithm using Robin software (Lohse et al., 2010), and differential expression was tested for the probesets called present in all chips (17,464 probesets according to the

present/absent MAS5 algorithm) correcting for multiple testing using the linear step-up false discovery rate (FDR). Changes in gene expression following the transfer to normal CO_2 conditions are reported as the difference between the $\log_2$ of relative expression levels after 2 days under normal CO_2 conditions and the $\log_2$ of relative expression levels at time zero (high CO_2).

For the metabolomic analysis, each metabolic-feature was normalized to the median within each experiment and genotype, and $\log_{10}$-transformed prior to statistical analysis. Statistical differences were assessed with two-way ANOVA at $P < 0.001$ using the Multiexperiment Viewer version 3.1 (Saeed et al., 2006). The variables "genotype" (wild type or mutant) and "time of exposure to normal CO_2" (0, 2, 3, 4,6, 8 10 days) were used as factors including all the independent samples. Data were $\log_{10}$-transformed only for the statistical analysis. The data presented in Fig. 6 represent the direct normalized responses of metabolite pool measures, that is, mass detector signals in arbitrary units normalized to internal standard and sample fresh weigh.

Numeric data for hierarchical clustering of the qRT-PCR data in Fig. 4 were obtained by subtracting the $\log_2$ of relative expression levels in the mutant from those of the WT. Hierarchical clustering of qRT-PCR data was performed using the Multiexperiment Viewer software version 4.8.1 (Saeed et al., 2006) with optimized gene leaf order and complete linkage clustering algorithm. Hierarchical clustering of the statistically significant MSTs was performed using both WT and mutant data and using Multiexperiment Viewer version 3.1 using a matrix based on Pearson correlation.

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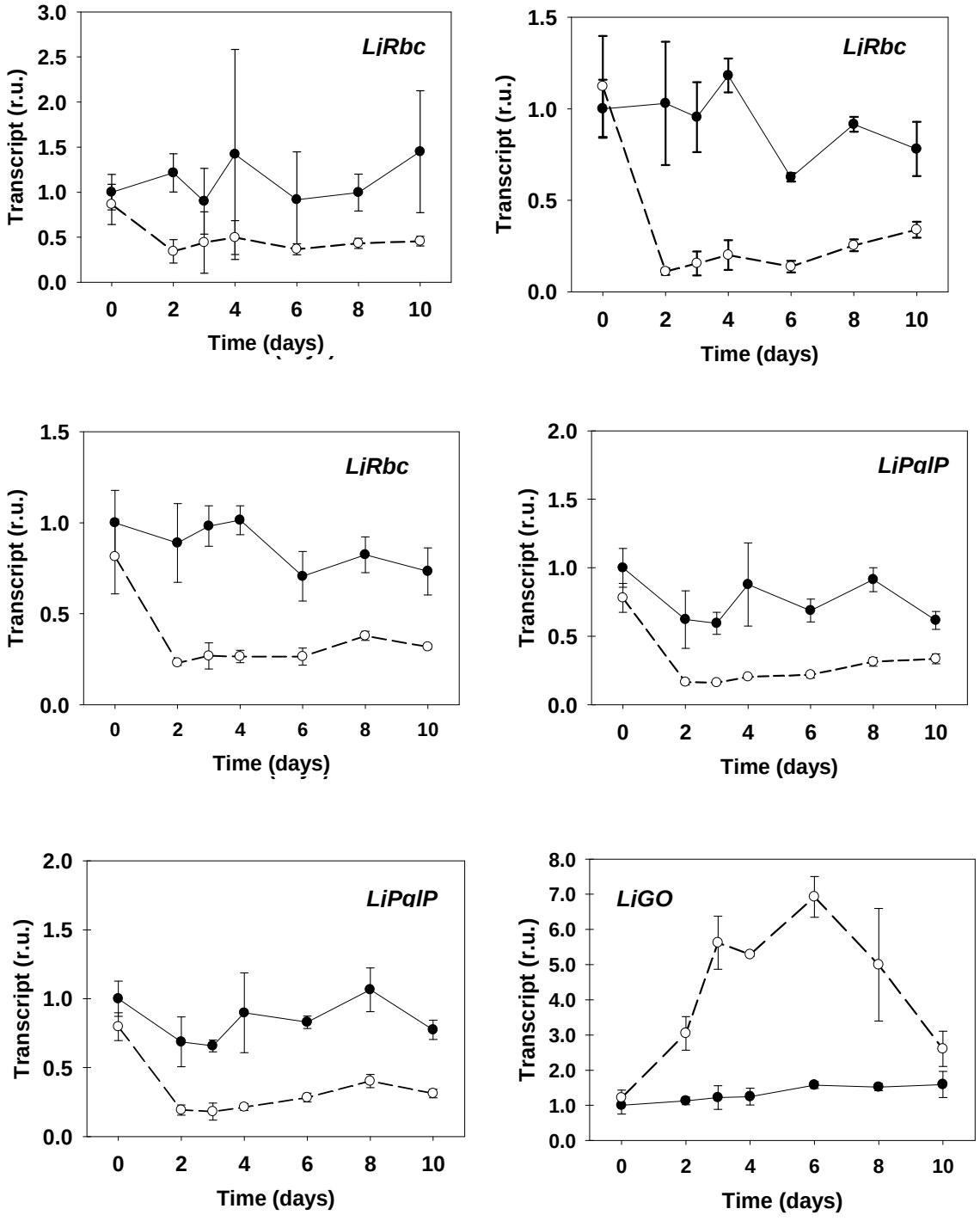
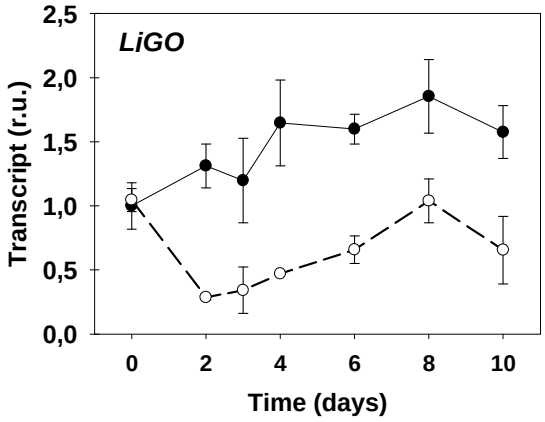
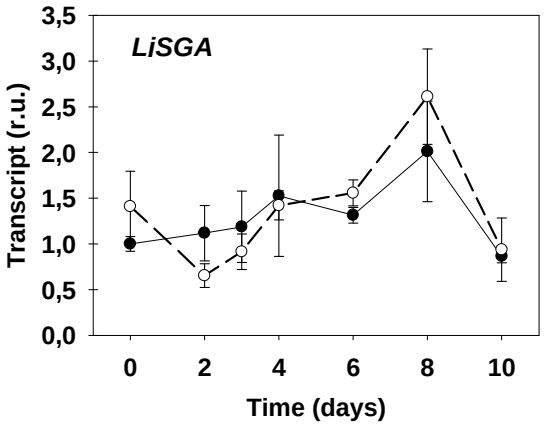
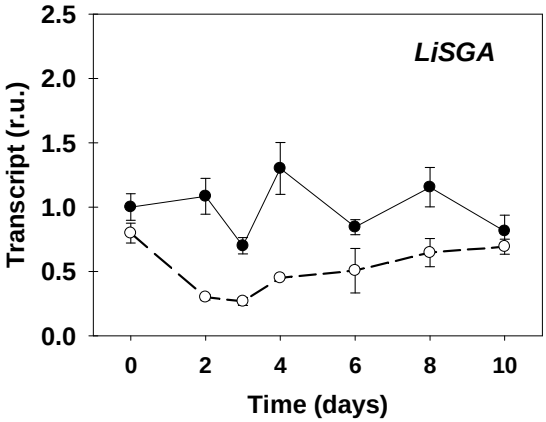
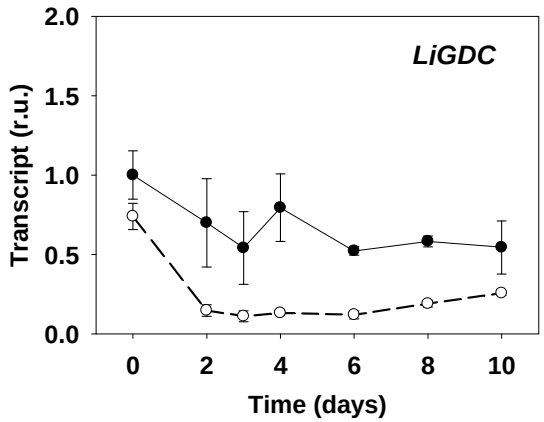
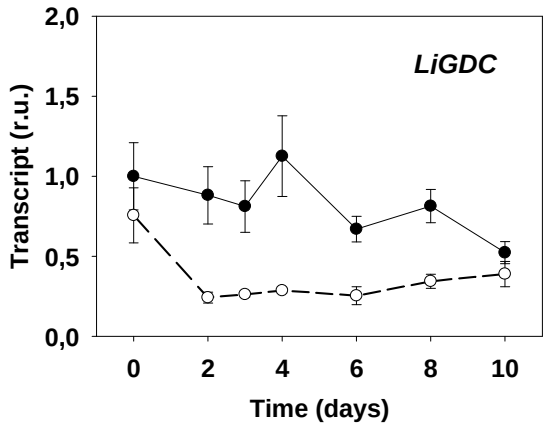


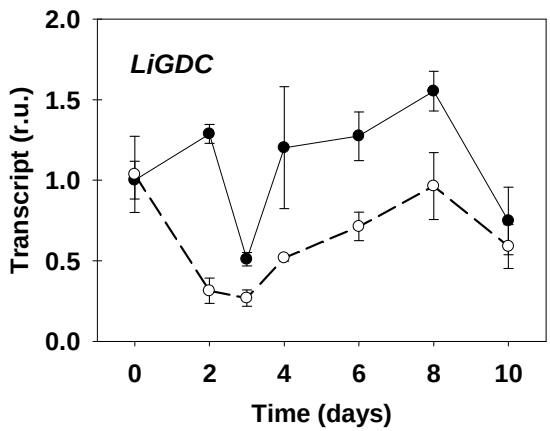

LiGO
Transcript (r.u.)
Time (days)

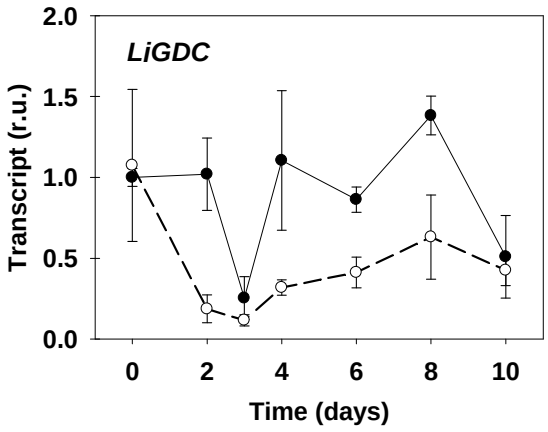

LiSGA
Transcript (r.u.)
Time (days)

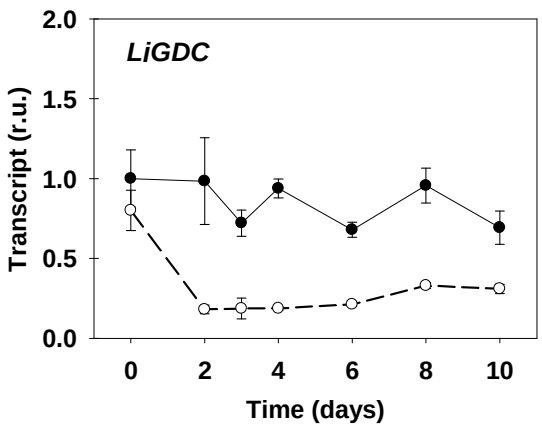

LiSGA
Transcript (r.u.)
Time (days)

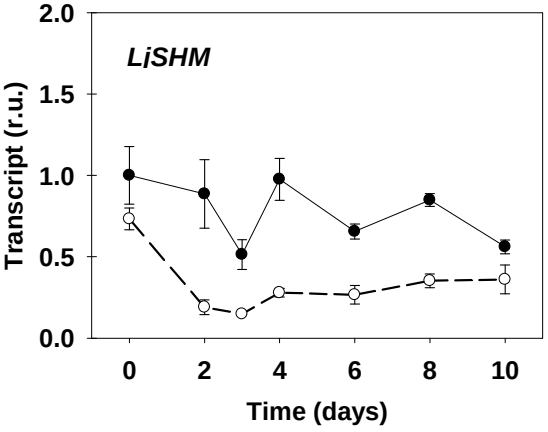

LiGDC
Transcript (r.u.)
Time (days)

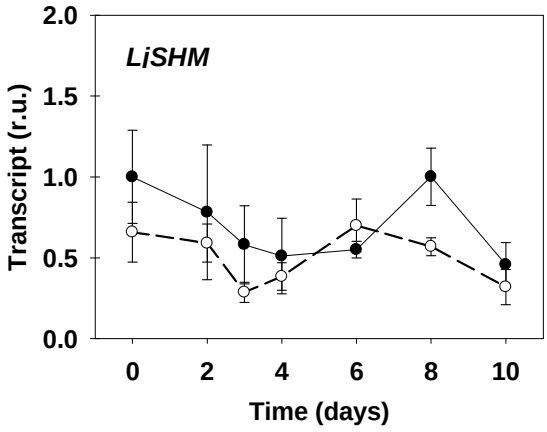

LiGDC
Transcript (r.u.)
Time (days)

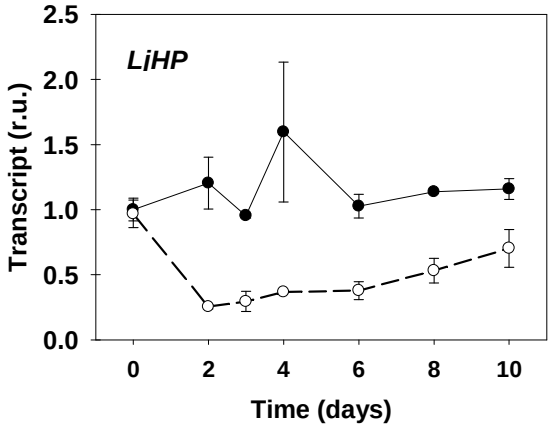

LiGDC
Transcript (r.u.)
Time (days)

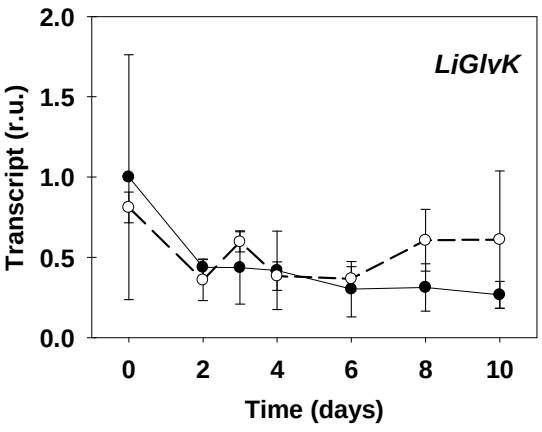

LiGDC
Transcript (r.u.)
Time (days)

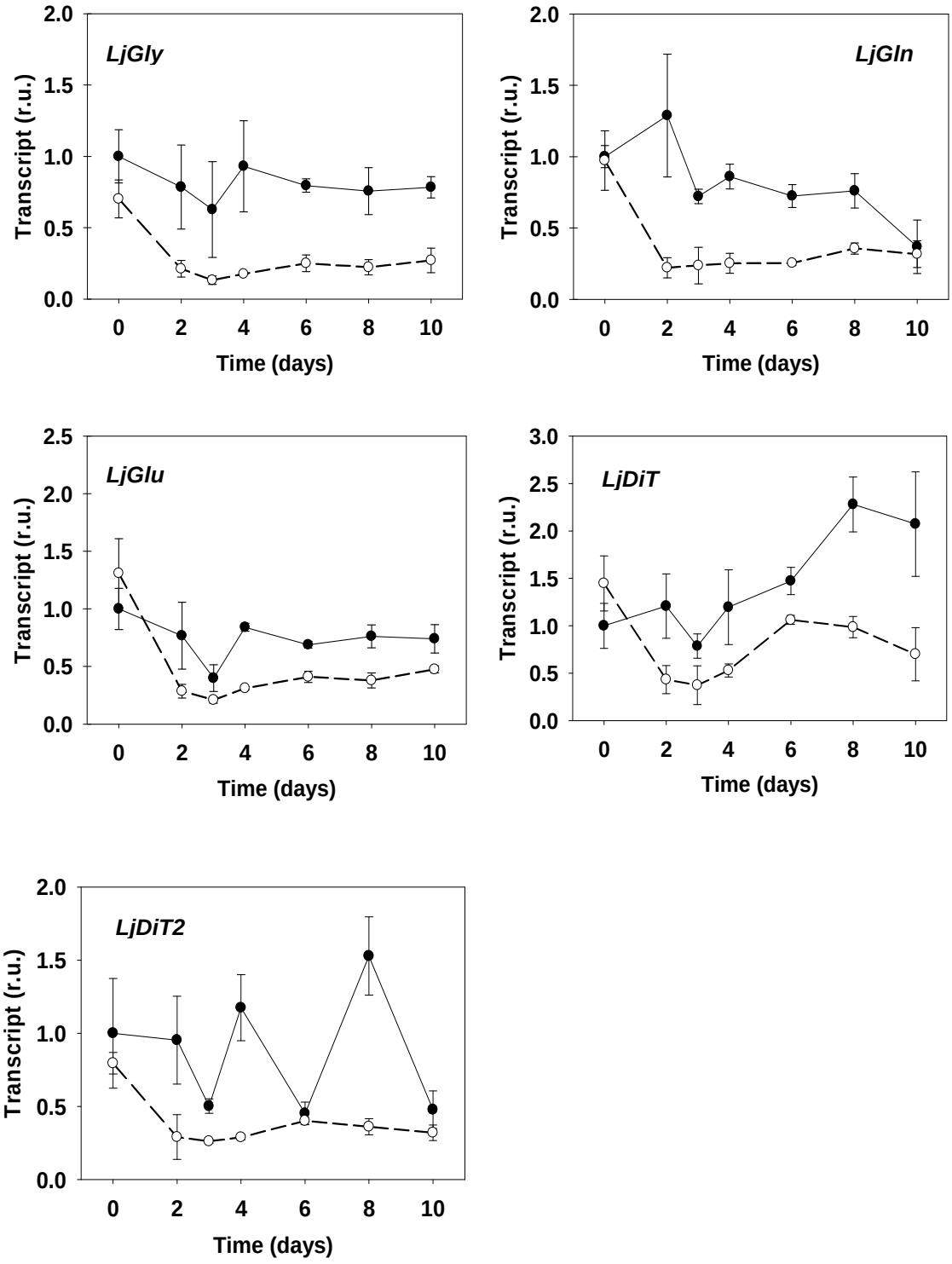

LiGDC
Transcript (r.u.)
Time (days)


LiSHM
Transcript (r.u.)
Time (days)


LiSHM
Transcript (r.u.)
Time (days)


LiHP
Transcript (r.u.)
Time (days)

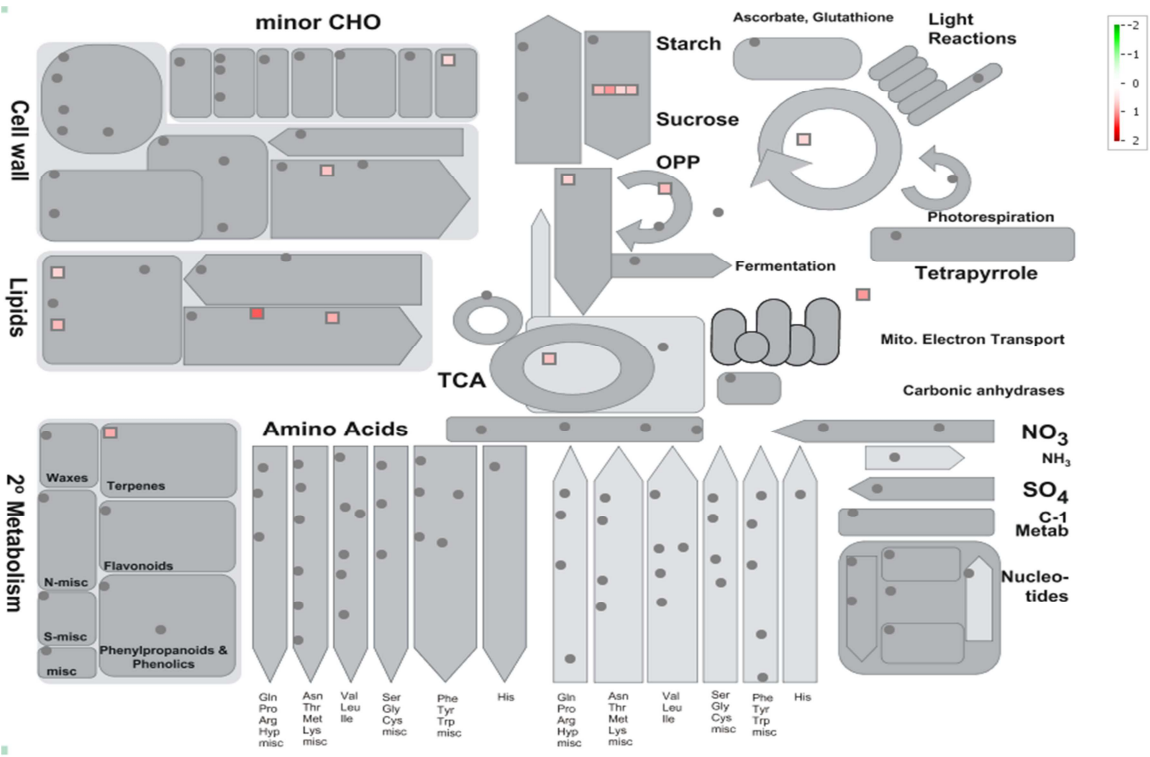

LiGlvK
Transcript (r.u.)
Time (days)



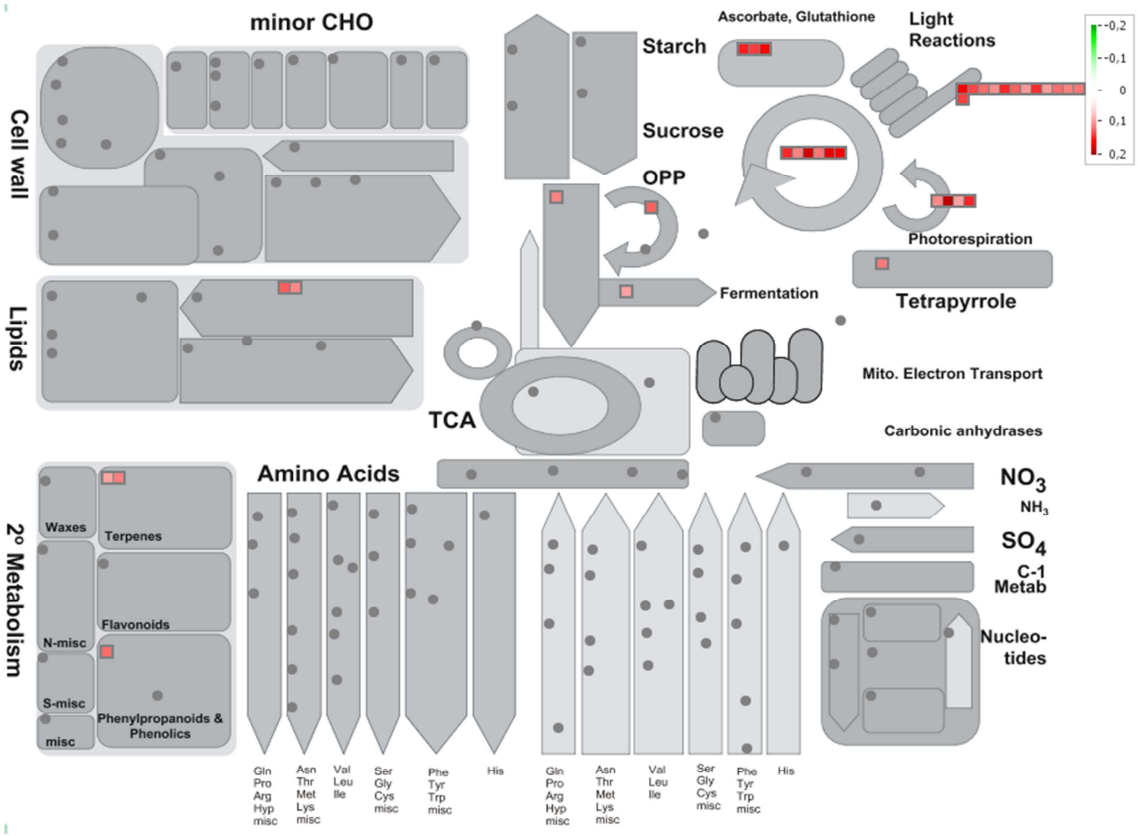
Supplemental Figure S1. Expression of photorespiratory genes in *Lotus japonicus* plants under active photorespiration. WT (solid line, black dots) and *Ljgln2-2* (dashed line, white dots) plants grown for 35 days in high CO_2 (time 0) were transferred to normal CO_2 conditions. Leaves were harvested at the indicated time points and the transcripts quantified by qRT-PCR. Transcript levels are reported as relative units (r.u.). For comparative purposes, the transcript levels measured in the WT plants under high CO_2 conditions (time 0) were taken as 1. The expression

of the following genes was studied: a gene for the RUBISCO large subunit (*LjRbc_l*, probeset *lj_rbcl*); two genes for the RUBISCO small subunit (*LjRbc_s1* and *LjRbc_s2*, probesets chr2.TM1655.9 and chr1.TM0220.16); two for phosphoglycolate phosphatase (*LjPgP1* and *LjPgP2*, probeset Ljwgs_028307.1 and TM0684.53); two for glycolate oxidase (*LjGO1* and *LjGO2*, probesets Ljwgs_013523.1 and Ljwgs_038509.1); two for serine:glyoxylate aminotransferase (*LjSGAT1* and *LjSGAT2*, probesets chr6.TM0085.11 and Ljwgs_079709.1); two for the H subunit of glycine decarboxylase (*LjGDCH1* and *LjGDCH2*, probesets Ljwgs_010991.0.1 and Ljwgs_087246.1); two for the P subunit of glycine decarboxylase (*LjGDCP1* and *LjGDCP2*, probesets chr5.CM0019.2 and chr5.CM0569.17.1); one for the T subunit of glycine decarboxylase (*LjGDCT*, probeset TM1304.24) two for serine:hydroxymethiltransferase (*LjSHM1* and *LjSHM2*, probesets Ljwgs_022333.1 and Ljwgs_020174.1); one for hydroxypyruvate reductase (*LjHPR*, probeset Ljwgs_011418.2); two for glycerate kinase (*LjGlyK1* and *LjGlyK2*, probesests chr3.CM0253.10.1 and chr3.CM0226.44); one for plastidic glutamine synthetase (*LjGln2*, probesets gi18266052 and TM1765.11); one for ferredoxin-dependent GOGAT (*LjGlu1*, probesets chr1.CM0009.24, Ljwgs_090201.1 and Ljwgs_068766.1) and two different isoforms of the plastidic dicarboxylate transporter: *LjDiT1* (probesets chr5.CM0089.81) and *LjDiT2.1* (probesets chr6.CM0055.63.1 and chr6.CM0055.71). Glutamate:glyoxylate aminotransferase (*LjGGAT*, probeset chr2.CM0088.66) transcript was not detectable. Data are the mean ± S.D. of three independent biological replicates.

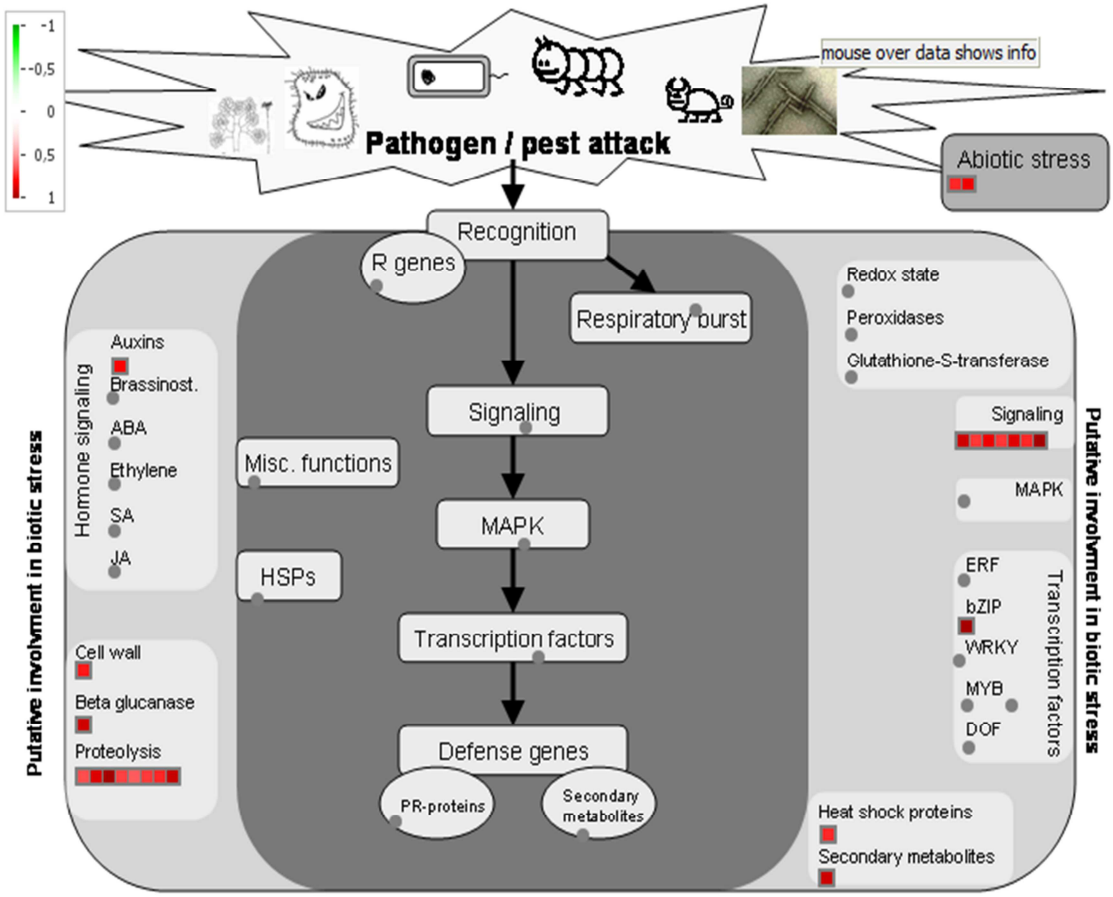
Supplemental Figure S2:

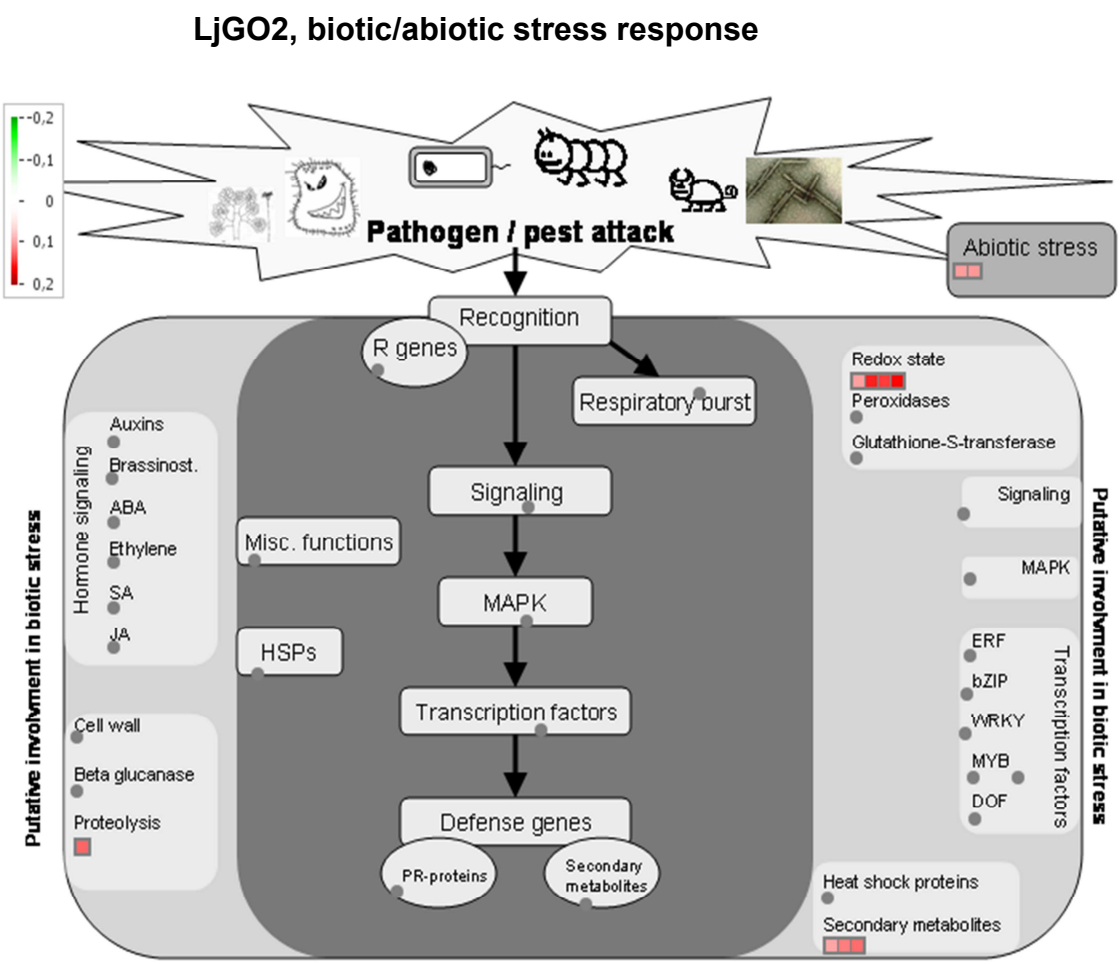


LjGO2, metabolism overview



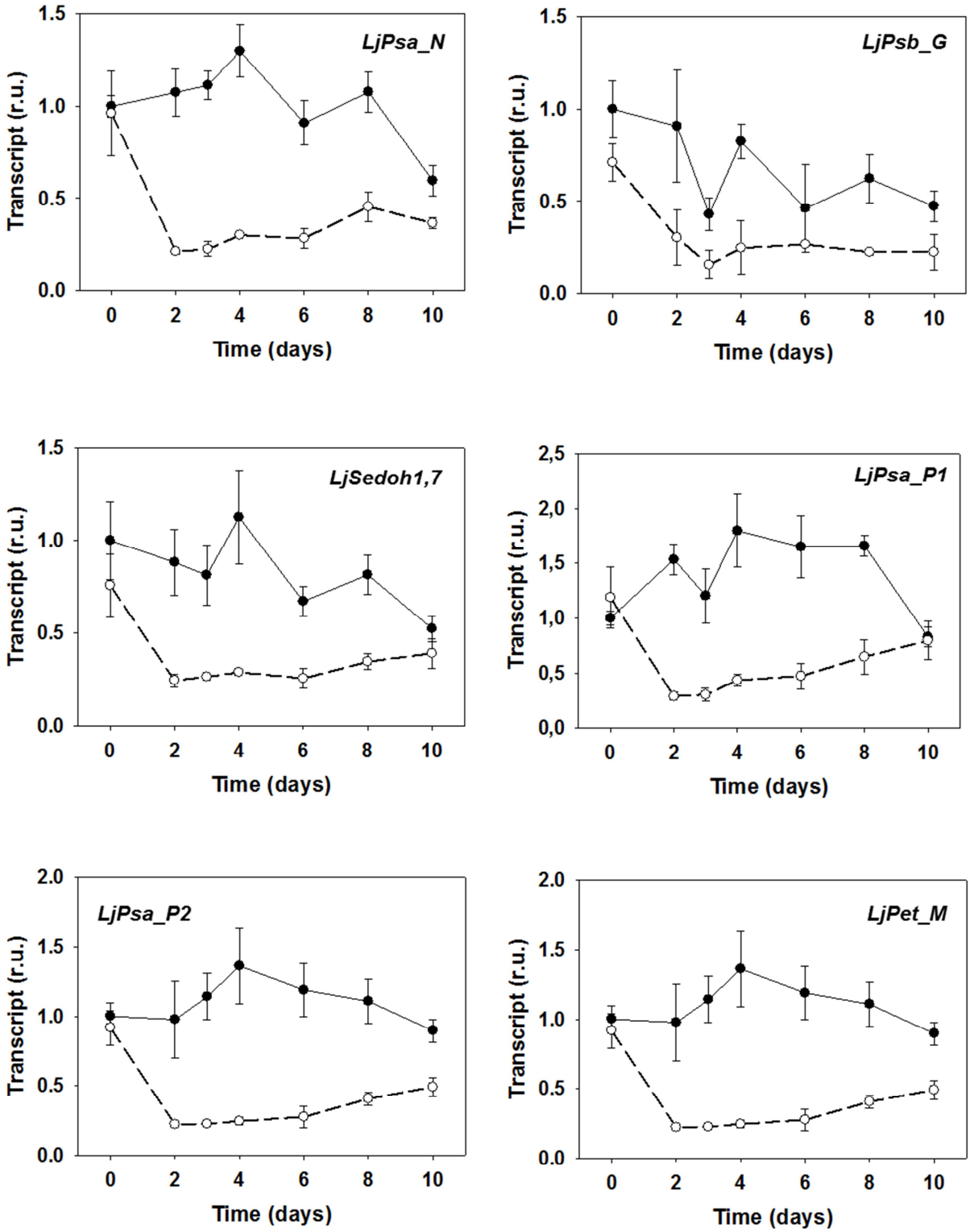
LiGO1. biotic/abiotic stress

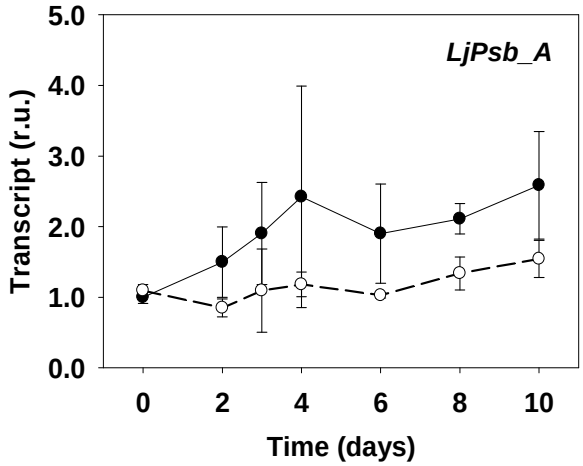




Supplemental Figure S2. Co-expression analysis of *LjGO1* and *LjGO2*. An overview of general metabolism and stress response was created using MapMan. Red squares represent genes that are positively co-expressed (Pearson distance <0.2) with the gene in question. The intensity of the red color is proportional to the degree of correlation between a certain gene and *LjGO1* or *LjGO2*. The scale bar is shown in $\log_2$. More details on data mining and analysis in Materials and Methods.

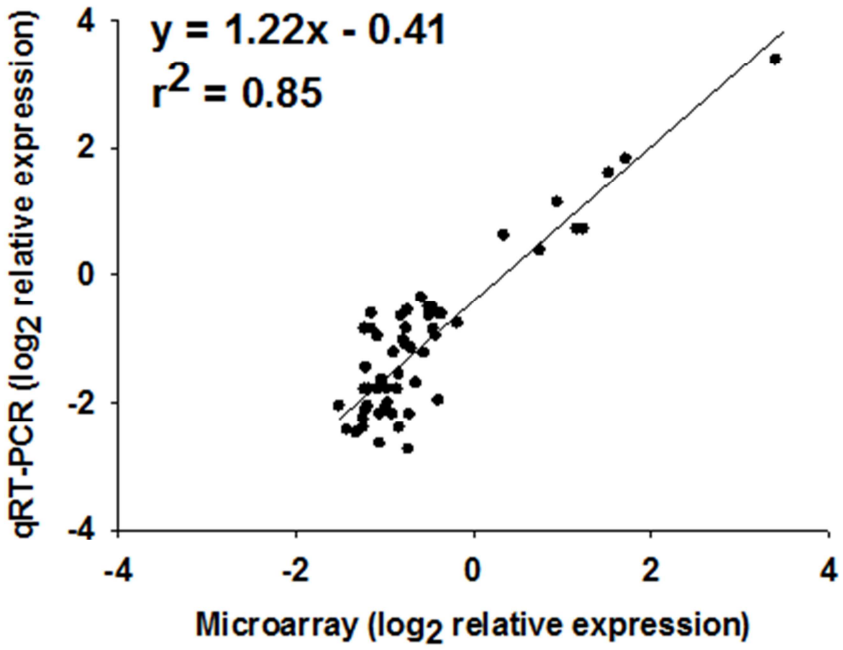
Supplemental Figure S3:





Supplemental Figure S3. Expression of genes encoding for some components of the photosynthetic apparatus in *Lotus japonicus* plants under active photorespiration. WT (solid line, black dots) and *Ljgln2-2* (dashed line, white dots). Plants grown for 35 days in high CO_2 (time 0) were transferred to normal CO_2 conditions. Leaves were harvested at the indicated time points and the different transcripts quantified by qRT-PCR. Transcript levels are reported as relative units (r.u.). For comparative purposes, the transcript levels measured in the WT plants under high CO_2 conditions (time 0) were taken as 1. The expression of the following genes was quantified: photosystem I reaction centre subunit N (*LjPsa_N*, probeset Ljwgs_047114.1); photosystem II reaction centre protein G (*LjPsb_G*, probeset Lj_PsbG): two isoform of the photosystem I P subunit (*LjPsa_P1* and *LjPsa_P2*, probesets chr1.CM0105.61 and chr3.CM0208.32 respectively); cytochrome b6f complex subunit (*LjPet_M*, probeset chr1.CM0523.11); photosystem II protein D1 (*LjPsb_A*, probeset Lj_psbA); sedoheptulose-1,7-bisphosphatase (*LjSedoh1,7*, probeset gi45411137). Data are the mean ± S.D. of three independent biological replicates.

Supplemental Figure S4:



Supplemental Figure S4. Validation of microarray data by qRT-PCR. A comparison of the $\log_2$ of the fold-change in relative gene expression levels after the transfer from high CO_2 to normal CO_2 conditions according to either qRT-PCR or microarray analysis is presented. The parameters obtained with a linear regression analysis of the data are reported in the inset. Only genes that were significantly modulated according to the Affychip were validated by qRT-PCR. 56 genes whose expression levels were determined to confirm the gene ontology analysis (Supplemental Table S2) or to study photosynthetic and photorespiratory metabolism (Supplemental Figs. S1 and S3, Table 1) were chosen for this study. Data are the mean of three independent biological replicates and include both WT and mutant genotypes.

Supplemental Figure S5:

WT only

Identification of represented BINs

p-value threshold: 0.05
Ajusted threshold: 6.02119460500963e-06

BIN				
BIN code	BIN name	Nb. of probe sets assigned	Nb. of probe sets submitted	p-value
99.1.2.60	COG2036 Histones H3 and H4	41	9	1.52969071033648e-09
99.1.4.5798	GO_ID:20630 TAIR|gene:2026493 SPTR:Q9LQQ4 symbol:AT1G07790.1 [GO:0003677 "DNA binding" evidence=ISS] InterPro:IPR000558 Interpro:IPR007125 Pfam:PF00125 InterPro:IPR007124 GO:GO:0005694 GO:GO:0005634 GO:GO:0000786 PRINTS:PR00621 ProDom:PD000497 PROSITE:PS00357 SMART:SM00427 EMBL:AC007583 EMBL:AY070760 EMBL:AY133638 EMBL:AF332446 PIR:D86213 HSSP:P02279 SMR:Q9LQQ4 SPTR:Q9LQQ4	4	4	2.285748045318 80e-08
99.1.3.2.2065	H3_WHEAT (P68428) Histone H3	9	5	3.38607558044733e-08
99.1.3.2.621	H2B_GOSHI (O22582) Histone H2B	3	3	1.863259077441 46e-06
99.1.4.1695	GO_ID:55079 MGI|MGI:2448329 SPTR:P84228 symbol:Hist1h3f "histone 1, H3f" [GO:0005694 "chromosome" evidence=RCA] [GO:0005634 "nucleus" evidence=RCA] [GO:0003677 "DNA binding" evidence=RCA] [GO:0007001 "chromosome organization and biogenesis (sensu Eukaryota)" evidence=RCA] [GO:0006334 "nucleosome assembly" evidence=RCA] [GO:0000786 "nucleosome" evidence=RCA] InterPro:IPR000164 Interpro:IPR007125 Pfam:PF00125 InterPro:IPR007124 MGI:MGI:2448319 MGI:MGI:2448329 MGI:MGI:2448357 InterPro:IPR009072 SMART:SM00428 PROSITE:PS00322 PROSITE:PS00959 EMBL:X01685 EMBL:X16148 EMBL:M32459 EMBL:M32461 EMBL:M33989 EMBL:U62669 EMBL:BC015270 PIR:JH0304 SMR:P84228 SPTR:P84228	18	5	2.10018162303870e-06
99.1.3.2.1332	H4_SILLA (Q76H85) Histone H4	10	4	4.52495045486684e-06
99.1.4.6252	GO_ID:51093 UNIPROT|P62801 SPTR:P62801 symbol:H4_CHICK "Histone H4" [GO:0003677 "DNA binding" evidence=ISS] [GO:0006323 "DNA packaging" evidence=ISS] [GO:0000786 "nucleosome" evidence=ISS] InterPro:IPR001951 Interpro:IPR007125 Pfam:PF00125 InterPro:IPR007124 InterPro:IPR009072 GO:GO:0000786 PROSITE:PS00047 EMBL:X02218 PDB:1EQZ PDB:1HIO PDB:1HQ3 PDB:1TZY PDB:2HIO EMBL:U37575 EMBL:J00866 EMBL:M74533 EMBL:M74534 PIR:A02640 PIR:JH0507 SMR:P62801 SPTR:P62801	10	4	4.52495045486684e-06
99.1.3.2.2216	RB11A_LOTJA (Q40191) Ras-related protein Rab11A	5	3	1.82914083962314e-05

Shared

Identification of represented BINs

p-value threshold: 0.05
Ajusted threshold: 6.02119460500963e-06

BIN			Nb. of probe sets submitted	p-value
BIN code	BIN name	Nb. of probe sets assigned		
1	Metabolism [CLASS:01100]	8170	266	6.40926016731394e-34
1.1	Carbohydrate Metabolism [CLASS:01110]	3640	143	1.51202672574607e-24
1.10	Biosynthesis of Secondary Metabolites [CLASS:01195]	1589	86	2.21888897577526e-23
1.3	Lipid Metabolism [CLASS:01130]	1589	73	3.85542912402581e-16
1.10.6	Stilbene, coumarine and lignin biosynthesis [CLASS:00940] [PATH:ot00940]	565	39	1.76173951865523e-14
1.11	Biodegradation of Xenobiotics [CLASS:01196]	1834	75	5.97982510335669e-14
1.5	Amino Acid Metabolism [CLASS:01150]	1941	76	3.52805088066522e-13
1.10.7	Flavonoid biosynthesis [CLASS:00941] [PATH:ot00941] [GO:0009813]	609	38	8.55260435383618e-13
1.1.7	Ascorbate and aldarate metabolism [CLASS:00053] [PATH:ot00053 tab00053]	416	27	7.35827712253893e-10
1.10.7.9	E1.14.13.21 flavonoid 3-monooxygenase [EC:1.14.13.21] [GO:0016711]	204	18	4.57311169474008e-09
1.3.3	Fatty acid metabolism [CLASS:00071] [PATH:ot00071 tab00071]	485	28	4.78524428252096e-09
1.11.4.6	E1.14.-.- [EC:1.14.-.-] [COG:COG2124]	212	18	8.386286552106e-09
1.11.14.4	E1.14.-.- [EC:1.14.-.-] [COG:COG2124]	212	18	8.386286552106e-09
1.10.6.12	E1.14.-.- [EC:1.14.-.-] [COG:COG2124]	212	18	8.386286552106e-09
1.10.3.12	E1.14.-.- [EC:1.14.-.-] [COG:COG2124]	212	18	8.386286552106e-09
1.1.7.13	E1.14.-.- [EC:1.14.-.-] [COG:COG2124]	212	18	8.386286552106e-09
1.3.3.23	E1.14.14.1 unspecific monooxygenase [EC:1.14.14.1] [COG:COG2124] [GO:0050381]	218	18	1.29746830086523e-08
1.5.14.29	E1.14.14.1 unspecific monooxygenase [EC:1.14.14.1] [COG:COG2124] [GO:0050381]	218	18	1.29746830086523e-08
1.11.4.2	E1.14.14.1 unspecific monooxygenase [EC:1.14.14.1] [COG:COG2124] [GO:0050381]	218	18	1.29746830086523e-08
1.9	Metabolism of Cofactors and Vitamins [CLASS:01190]	1962	65	1.52848668869172e-08
1.11.4	gamma-Hexachlorocyclohexane degradation [CLASS:00361] [PATH:ot00361]	340	22	2.8716288531353e-08
1.11.14	Fluorene degradation [CLASS:00628] [PATH:ot00628]	263	19	4.46053050478625e-08
1.7	Glycan Biosynthesis and Metabolism [CLASS:01170]	1671	56	1.08186406033769e-07
1.10.3	Limonene and pinene degradation [CLASS:00903] [PATH:ot00903]	337	21	1.11267786993739e-07
1.1.8.12	E3.2.1.39 glucan endo-1,3-beta-D-glucosidase [EC:3.2.1.39]	80	10	5.02024154749746e-07
1.6	Metabolism of Other Amino Acids [CLASS:01160]	726	31	6.57107967843453e-07
1.2.6	Methane metabolism [CLASS:00680] [PATH:ot00680 tab00680]	229	16	7.94567112089132e-07
1.1.8	Starch and sucrose metabolism [CLASS:00500] [PATH:ot00500 tab00500]	1062	39	1.10311044793362e-06
1.5.14	Tryptophan metabolism [CLASS:00380] [PATH:ot00380]	578	26	2.02426583464059e-06
1.5.13.21	E1.11.1.7 peroxidase [EC:1.11.1.7] [GO:0004601]	143	12	2.85543323104696e-06
1.3.11.18	E1.11.1.7 peroxidase [EC:1.11.1.7] [GO:0004601]	143	12	2.85543323104696e-06
1.10.6.4	E1.11.1.7 peroxidase [EC:1.11.1.7] [GO:0004601]	143	12	2.85543323104696e-06
1.2.6.9	E1.11.1.7 peroxidase [EC:1.11.1.7] [GO:0004601]	143	12	2.85543323104696e-06
1.5.13	Phenylalanine metabolism [CLASS:00360] [PATH:ot00360 tab00360]	525	24	3.77427910420494e-06
99.1.3.2.2117	PR1_MEDSA (Q43560) Class-10 pathogenesis-related protein 1 (MSPR10-1)	8	4	3.93082261642529e-06
1.9.4	Nicotinate and nicotinamide metabolism [CLASS:00760] [PATH:ot00760 tab00760]	972	35	5.93927771384592e-06
1.7.8	Lipopolysaccharide biosynthesis [CLASS:00540] [PATH:ot00540 tab00540]	1198	39	1.78725460284643e-05

LjgIn2-2
only

Identification of represented BINs

p-value threshold: 0.05
Ajusted threshold: 6.02119460500963e-06

BIN				
BIN code	BIN name	Nb. of probe sets assigned	Nb. of probe sets submitted	p-value
1	Metabolism [CLASS:01100]	8170	1544	4.71124636943774e-125
1.1	Carbohydrate Metabolism [CLASS:01110]	3640	704	9.21266411566154e-56
1.9	Metabolism of Cofactors and Vitamins [CLASS:01190]	1962	445	2.87897696605614e-53
1.5	Amino Acid Metabolism [CLASS:01150]	1941	433	8.39023353368761e-50
1.11	Biodegradation of Xenobiotics [CLASS:01196]	1834	390	8.61402191448816e-40
1.10	Biosynthesis of Secondary Metabolites [CLASS:01195]	1589	324	1.17999207946585e-29
1.3	Lipid Metabolism [CLASS:01130]	1589	312	1.01637403917933e-25
1.2	Energy Metabolism [CLASS:01120]	1367	272	2.04309082684327e-23
1.9.10	Porphyrin and chlorophyll metabolism [CLASS:00860] [PATH:ot00860 tab00860]	366	109	2.81178307018105e-23
1.6	Metabolism of Other Amino Acids [CLASS:01160]	726	168	1.30593924140772e-21
1.1.1	Glycolysis / Gluconeogenesis [CLASS:00010] [PATH:ot00010 tab00010] [GO:0006096 0006094]	477	124	1.18590878177591e-20
1.2.4	Carbon fixation [CLASS:00710] [PATH:ot00710]	194	70	1.47560713970459e-20
1.11.16	Benzoate degradation via CoA ligation [CLASS:00632] [PATH:ot00632]	997	207	2.75726645377912e-20
1.1.3	Pentose phosphate pathway [CLASS:00030] [PATH:ot00030 tab00030] [GO:0006098]	248	80	8.39410405923408e-20
1.7	Glycan Biosynthesis and Metabolism [CLASS:01170]	1671	302	2.64088222656495e-19
1.9.4	Nicotinate and nicotinamide metabolism [CLASS:00760] [PATH:ot00760 tab00760]	972	200	3.42572969195453e-19
3	Environmental Information Processing [CLASS:01300]	3996	606	1.88162939792225e-18
3.2	Signal Transduction [CLASS:01320]	2883	461	3.75392732328016e-18
1.7.8	Lipopolysaccharide biosynthesis [CLASS:00540] [PATH:ot00540 tab00540]	1198	229	9.48022329012102e-18
1.1.12	Glyoxylate and dicarboxylate metabolism [CLASS:00630] [PATH:ot00630 tab00630]	207	68	1.58771292189743e-17
99.1.2	Unclassified with homolog in COG	866	176	1.63129546587381e-16
1.1.5	Fructose and mannose metabolism [CLASS:00051] [PATH:ot00051 tab00051]/ [GO:0006000 0006013]	367	95	4.77267092751965e-16
1.10.7	Flavonoid biosynthesis [CLASS:00941] [PATH:ot00941] [GO:0009813]	609	133	2.73820733167878e-15
1.5.3	Glycine, serine and threonine metabolism [CLASS:00260] [PATH:ot00260 tab00260]	305	81	1.44427398751495e-14
1.5.13	Phenylalanine metabolism [CLASS:00360] [PATH:ot00360 tab00360]	525	117	2.87095535905212e-14
1.9.4.15	E2.7.1.- [EC:2.7.1.-] [COG:COG0515 COG1493]	825	161	1.1651530045854e-13
1.1.17.19	E2.7.1.- [EC:2.7.1.-] [COG:COG0515 COG1493]	825	161	1.1651530045854e-13
1.7.8.19	WAAY, rfaY lipopolysaccharide core biosynthesis protein RFAY [EC:2.7.-.-] [COG:COG0515]	825	161	1.1651530045854e-13
1.11.16.8	E2.7.1.- [EC:2.7.1.-] [COG:COG0515 COG1493]	825	161	1.1651530045854e-13
3.2.9	Calcium signaling pathway [CLASS:04020] [PATH:hsa04020] [GO:0019722]	474	107	1.48412346363010e-13
1.5.14	Tryptophan metabolism [CLASS:00380] [PATH:ot00380]	578	123	2.06387881053518e-13
1.1.13	Propanoate metabolism [CLASS:00640] [PATH:ot00640 tab00640]	253	69	3.13373683032041e-13
1.1.11	Pyruvate metabolism [CLASS:00620] [PATH:ot00620 tab00620] [GO:0006090]	366	88	5.3117138268809e-13
1.5.10	Arginine and proline metabolism [CLASS:00330] [PATH:ot00330 tab00330]	340	83	1.00917604333211e-12
1.1.16	Inositol metabolism [CLASS:00031] [PATH:ot00031 tab00031]	119	42	1.43646214152755e-12
1.1.17	Inositol phosphate metabolism [CLASS:00562] [PATH:ot00562]	983	179	2.92092200201374e-12
1.9.5	Pantothenate and CoA biosynthesis [CLASS:00770] [PATH:ot00770 tab00770]	88	34	1.03054654754098e-11
1.5.5	Cysteine metabolism [CLASS:00272] [PATH:ot00272 tab00272]	93	35	1.24590065323572e-11
3.2.11	Insulin signaling pathway [CLASS:04910] [PATH:hsa04910]	640	126	2.85714461886359e-11

LjgIn2-2 only (follows from previous page)

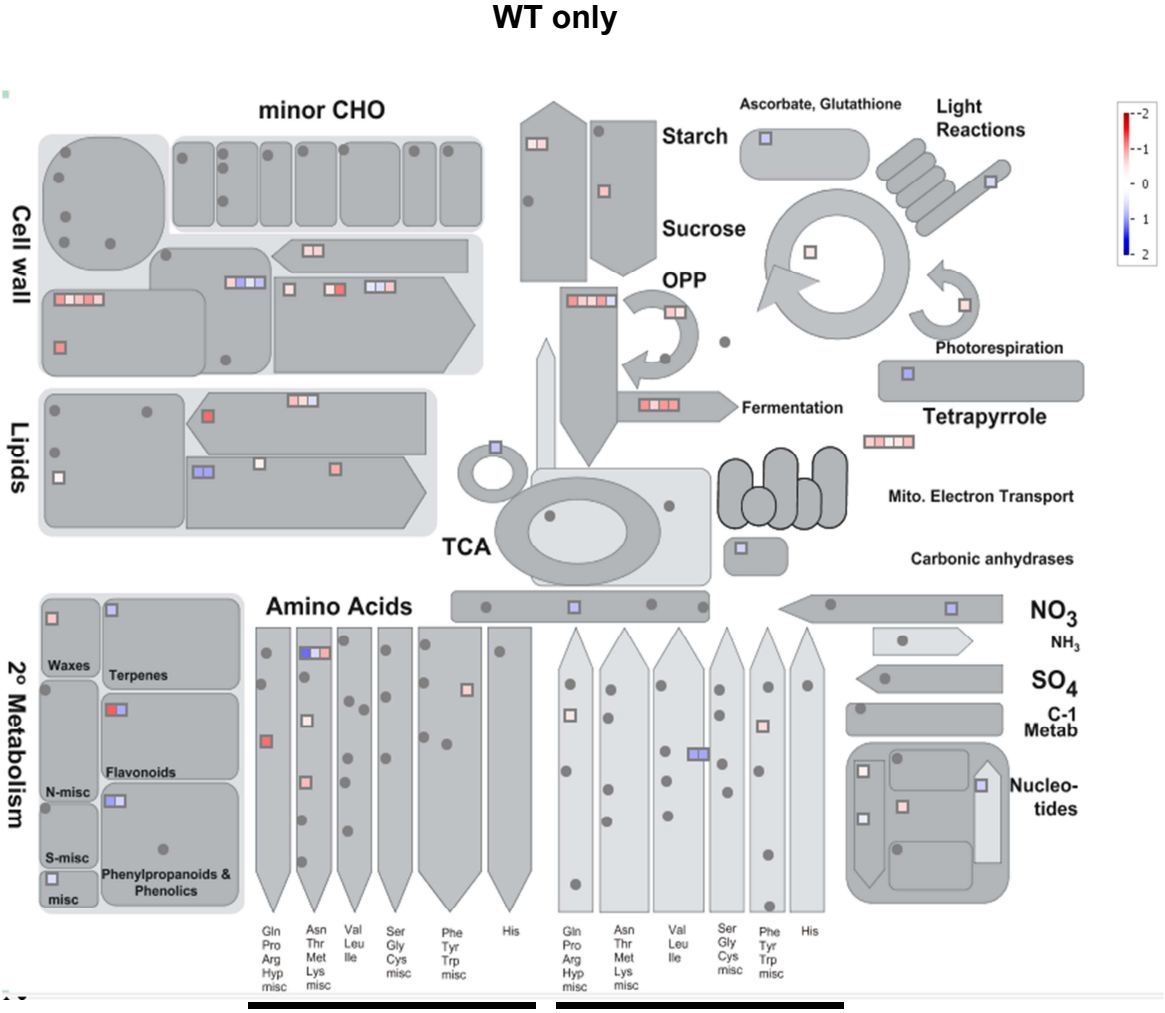
1.1.14	Butanoate metabolism [CLASS:00650] [PATH:ot00650 tab00650]	377	85	4.74512028095405e-11
3.2.2	MAPK signaling pathway [CLASS:04010] [PATH:hsa04010]	1569	254	4.80526589957244e-11
1.5.11	Histidine metabolism [CLASS:00340] [PATH:ot00340 tab00340]	240	62	5.81280640721642e-11
1.5.1	Glutamate metabolism [CLASS:00251] [PATH:ot00251 tab00251]	283	69	8.31006950345773e-11
1.3.3	Fatty acid metabolism [CLASS:00071] [PATH:ot00071 tab00071]	485	101	1.01361217172741e-10
1.5.6	Valine, leucine and isoleucine degradation [CLASS:00280] [PATH:ot00280 tab00280]	223	58	1.78053644717106e-10
1.11.17	Benzoate degradation via hydroxylation [CLASS:00362] [PATH:ot00362]	197	53	2.81496169674952e-10
1.3.6	Bile acid biosynthesis [CLASS:00120] [PATH:ot00120]	197	53	2.81496169674952e-10
2.3.1	Protein folding and associated processing [CLASS:03100]	725	135	2.95935614018676e-10
1.5.7	Valine, leucine and isoleucine biosynthesis [CLASS:00290] [PATH:ot00290 tab00290]	150	44	4.57207164403554e-10
1.11.12	Nitrobenzene degradation [CLASS:00626] [PATH:ot00626]	145	43	4.91041022484985e-10
1.5.15	Phenylalanine, tyrosine and tryptophan biosynthesis [CLASS:00400] [PATH:ot00400 tab00400]	147	43	7.88659900178157e-10
1.1.16.2	myo-inositol catabolism protein	52	23	9.41420291930034e-10
99.1.1	Unclassified with homolog in KEGG (KO)	1124	186	3.49284989386607e-09
1.11.10	Styrene degradation [CLASS:00643] [PATH:ot00643]	151	42	6.49810060625715e-09
1.5.9	Lysine degradation [CLASS:00310] [PATH:ot00310]	246	58	9.45003332110514e-09
2.3	Folding, Sorting and Degradation [CLASS:01230]	1515	234	1.82899643258775e-08
1.9.1	Thiamine metabolism [CLASS:00730] [PATH:ot00730 tab00730]	51	21	2.37607919170923e-08
3.2.9.88	CYPD, PPID peptidylprolyl isomerase D (cyclophilin D) [EC:5.2.1.8] [GO:0016018]	52	21	3.57731842739535e-08
1.11.20	1- and 2-Methylnaphthalene degradation [CLASS:00624] [PATH:ot00624]	189	47	4.07245311151185e-08
1.6.1	beta-Alanine metabolism [CLASS:00410] [PATH:ot00410 tab00410]	207	50	4.20138180855216e-08
1.10.7.1	E2.3.1.74, bcsA chalcone synthase [EC:2.3.1.74] [COG:COG3424] [GO:0016210]	57	22	4.52342739067361e-08
1.2.7	Nitrogen metabolism [CLASS:00910] [PATH:ot00910 tab00910]	327	69	4.96856823108873e-08
3.3.10.117	BMPR1 bone morphogenetic protein receptor, type I [EC:2.7.1.37]	1064	172	6.89482571539358e-08
1.11.4	gamma-Hexachlorocyclohexane degradation [CLASS:00361] [PATH:ot00361]	340	70	1.09570870282249e-07
1.6.3	Aminophosphonate metabolism [CLASS:00440] [PATH:ot00440]	154	40	1.14708762279321e-07
1.3.5	Biosynthesis of steroids [CLASS:00100] [PATH:ot00100 tab00100]	173	43	1.52204224756765e-07
1.10.16	Novobiocin biosynthesis [CLASS:00401] [PATH:ot00401]	52	20	1.96580291822537e-07
1.3.9	Glycerolipid metabolism [CLASS:00561] [PATH:ot00561 tab00561] [GO:0045017 0046486 0046503]	516	95	2.02500194138988e-07
3.2.11.9	E2.4.1.11, GYS glycogen(starch) synthase [EC:2.4.1.11] [COG:COG0297] [GO:0004373] [CAZy:GT3 GT5]	12	9	3.35887089481693e-07
1.1.8.45	E2.4.1.11, GYS glycogen(starch) synthase [EC:2.4.1.11] [COG:COG0297] [GO:0004373] [CAZy:GT3 GT5]	12	9	3.35887089481693e-07
1.10.3	Limonene and pinene degradation [CLASS:00903] [PATH:ot00903]	337	68	3.60404078855464e-07
1.6.4	Selenoamino acid metabolism [CLASS:00450] [PATH:ot00450]	143	37	3.72493862288465e-07
99.1.2.149	COG1100 GTPases	121	33	4.24328548410587e-07
4.2	Cell Growth and Death [CLASS:01420]	2118	302	4.31913239112347e-07
99.1.3	Unclassified with homolog in Swiss-Prot	8528	1057	4.53932811571202e-07
1.9.1.9	E2.7.1.89 thiamine kinase [EC:2.7.1.89] [COG:COG0510] [GO:0019165]	33	15	5.10927617437652e-07
1.1.7	Ascorbate and aldarate metabolism [CLASS:00053] [PATH:ot00053 tab00053]	416	79	5.83246080298138e-07
1.10.16.3	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.5.5.10	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.10.8.1	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.2.4.14	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.5.2.1	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.5.1.10	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.5.10.13	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07

Ljgln2-2 only (follows from previous page)

1.5.15.22	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.5.13.4	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.5.12.1	E2.6.1.1 aspartate aminotransferase [EC:2.6.1.1] [GO:0004069]	46	18	5.88913330738953e-07
1.1.8	Starch and sucrose metabolism [CLASS:00500] [PATH:ot00500 tab00500]	1062	167	6.3374318981589e-07
1.9.5.1	E2.2.1.6 acetolactate synthase [EC:2.2.1.6] [GO:0003984]	10	8	7.04076499422064e-07
1.1.14.41	E2.2.1.6 acetolactate synthase [EC:2.2.1.6] [GO:0003984]	10	8	7.04076499422064e-07
1.1.15.1	E2.2.1.6 acetolactate synthase [EC:2.2.1.6] [GO:0003984]	10	8	7.04076499422064e-07
1.5.7.1	E2.2.1.6 acetolactate synthase [EC:2.2.1.6] [GO:0003984]	10	8	7.04076499422064e-07
3.3.10	CD molecules [CLASS:04090] [FC:ko04090]	1234	189	7.37490449987554e-07
1.1.2	Citrate cycle (TCA cycle) [CLASS:00020] [PATH:ot00020 tab00020] [GO:0006099]	108	30	9.1260149509049e-07
1.10.12.3	E5.4.2.2, pgm phosphoglucomutase [EC:5.4.2.2] [COG:COG0033] [GO:0004614]	8	7	1.27966494739660e-06
1.1.1.33	E5.4.2.2, pgm phosphoglucomutase [EC:5.4.2.2] [COG:COG0033] [GO:0004614]	8	7	1.27966494739660e-06
1.1.3.18	E5.4.2.2, pgm phosphoglucomutase [EC:5.4.2.2] [COG:COG0033] [GO:0004614]	8	7	1.27966494739660e-06
1.1.6.5	E5.4.2.2, pgm phosphoglucomutase [EC:5.4.2.2] [COG:COG0033] [GO:0004614]	8	7	1.27966494739660e-06
1.1.8.33	E5.4.2.2, pgm phosphoglucomutase [EC:5.4.2.2] [COG:COG0033] [GO:0004614]	8	7	1.27966494739660e-06
1.1.8.41	E2.4.1.21, glgA starch synthase [EC:2.4.1.21] [COG:COG0297] [GO:0009011] [CAZy:GT5]	31	14	1.35382459230378e-06
3.3	Ligand-Receptor Interaction [CLASS:01330]	1383	206	1.70805811118998e-06
1.2.3	Photosynthesis [CLASS:00195] [PATH:ot00195 tab00195]	129	33	2.03314218665378e-06
1.3.10	Glycerophospholipid metabolism [CLASS:00564] [PATH:ot00564 tab00564] [GO:0006650 0046474 0046475]	307	61	2.35976232697993e-06
99.1.1.65	KO:K07976	96	27	2.41442187989822e-06
1.3.1	Fatty acid biosynthesis [CLASS:00061] [PATH:ot00061 tab00061]	130	33	2.44272985897035e-06
1.9.2	Riboflavin metabolism [CLASS:00740] [PATH:ot00740 tab00740]	60	20	2.76666207337923e-06
1.10.7.6	E2.4.1.91 flavonol 3-O-glucosyltransferase [EC:2.4.1.91] [GO:0047893] [CAZy:GT1]	131	33	2.92729214076693e-06
4.5	Development [CLASS:01440]	276	56	3.07732477393921e-06
1.5.8	Lysine biosynthesis [CLASS:00300] [PATH:ot00300 tab00300]	138	34	3.55956001002136e-06
99.1.3.1	Unclassified with homolog in Swiss-Prot (EC)	1504	219	4.02305526954378e-06
1.1.12.36	E4.1.1.8 oxalyl-CoA decarboxylase [EC:4.1.1.8] [COG:COG0028] [GO:0008949]	9	7	5.21316816830514e-06
1.1.15.1.1	E2.2.1.6L, ilvB acetolactate synthase large subunit [COG:COG0028]	9	7	5.21316816830514e-06
1.5.7.1.1	E2.2.1.6L, ilvB acetolactate synthase large subunit [COG:COG0028]	9	7	5.21316816830514e-06
1.9.5.1.1	E2.2.1.6L, ilvB acetolactate synthase large subunit [COG:COG0028]	9	7	5.21316816830514e-06
1.1.14.41.1	E2.2.1.6L, ilvB acetolactate synthase large subunit [COG:COG0028]	9	7	5.21316816830514e-06
1.2.3.1	Photosystem II	30	13	5.70539780295725e-06
1.11.1	Caprolactam degradation [CLASS:00930] [PATH:ot00930]	147	35	5.8830640925445e-06
1.1.15	C5-Branched dibasic acid metabolism [CLASS:00660] [PATH:ot00660]	19	10	8.20476829587559e-06

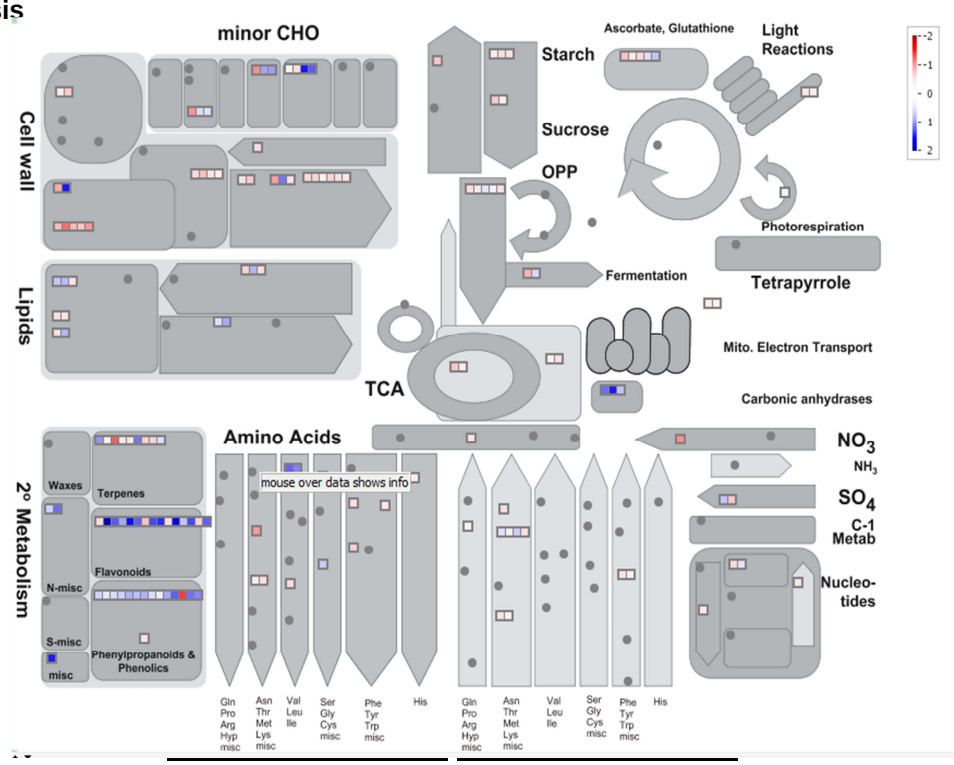
Supplemental Figure S5. Genebins analysis of over-represented BINs. The over-represented BINs among the probesets modulated by the transfer from high CO_2 to normal CO_2 conditions were obtained using the program Genebins. Genes elicited exclusively in WT plants (WT only), in both genotypes (Shared) and exclusively in the mutant (*Ljgln2-2* only) were analysed separately. The significant BINs ($P < 0.05$, Bonferroni correction) are highlighted in red.

Supplemental Figure S6:



WT

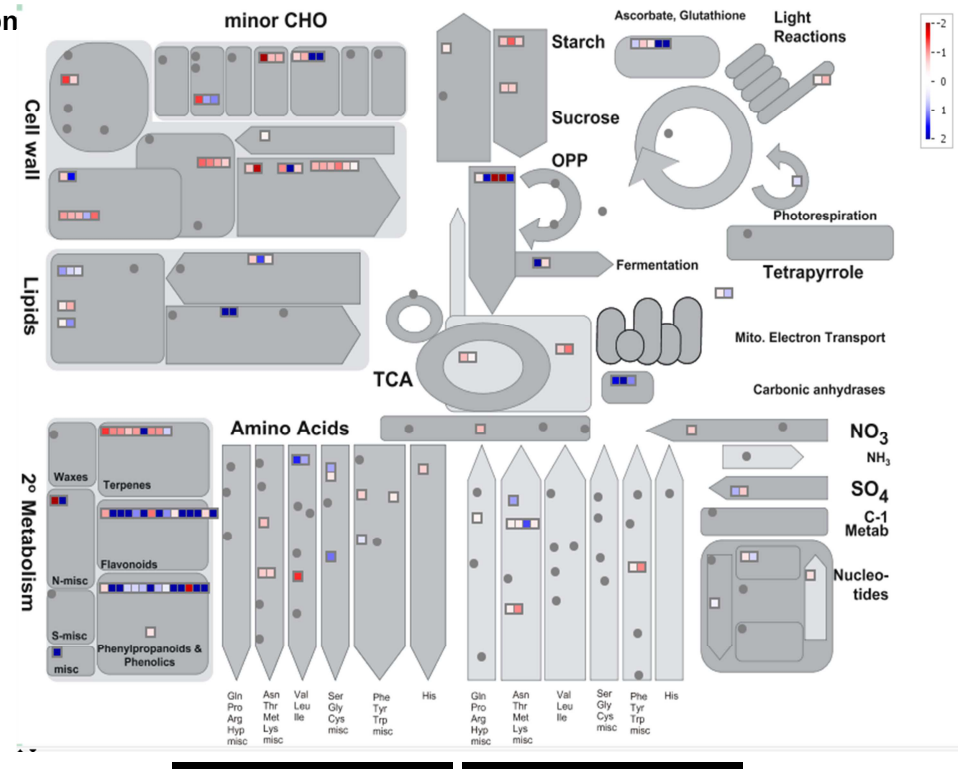
Amino acid synthesis



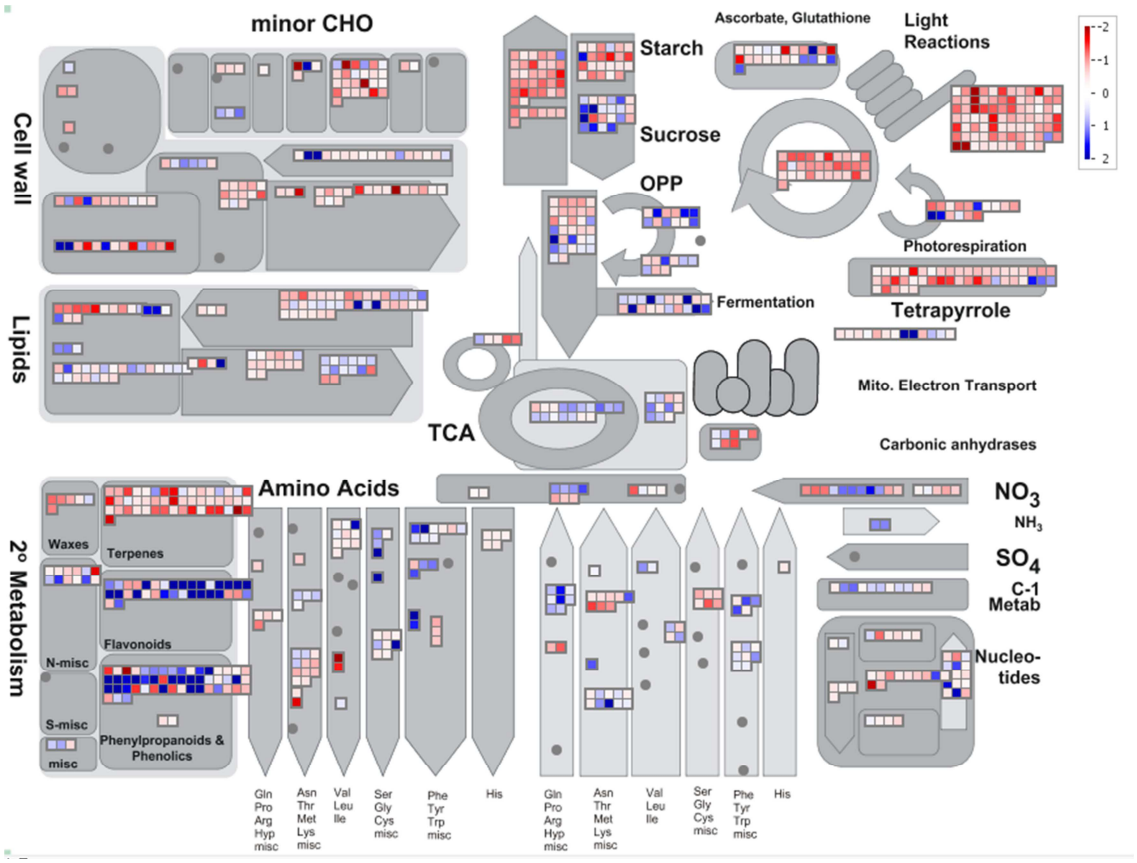
Ljgln2-2

WT and *Ljgln2-2*, shared

Amino acid degradation



LjgIn2-2 only



Amino acid synthesis | **Amino acid degradation**

Supplemental Figure S6. MapMan metabolism overview of probesets elicited by the transfer from high CO_2 to normal CO_2 conditions. The genes modulated exclusively in WT plants, in both genotypes and exclusively in the mutant are reported in each slide. Each square corresponds to a gene. Red and blue indicate higher and lower expression than the control, respectively. The scale bar is shown in $\log_2$.

Supplemental Figure S7:

Parameters

Genome Array:	Lotus Genome Array (Lotus japonicus)
Nb. of Identifiers submitted:	644
EC assignment:	78 EC <-> 123 identifiers view
Threshold:	0.1
Adjustment method:	fdr

Results

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted	P-value	Adjusted p-value
D-Arginine and D-ornithine metabolism	map	2	2	0.00873789283492787	0.265745095697850
Flavonoid biosynthesis	map	15	5	0.00901147515681421	0.265745095697850
Stilbene, coumarine and lignin biosynthesis	map	10	4	0.00972238154992136	0.265745095697850
Starch and sucrose metabolism	map	31	7	0.0198654086968359	0.407240878285136
Phenylalanine metabolism	map	14	4	0.034719484734939	0.551237562855165

Parameters

Amin

Genome Array:	Lotus Genome Array (Lotus japonicus)
Nb. of Identifiers submitted:	816
EC assignment:	133 EC <-> 258 identifiers view
Threshold:	0.1
Adjustment method:	fdr

Results

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted	P-value	Adjusted p-value
Starch and sucrose metabolism	map	31	16	3.15935097014919e-06	0.000274863534402980
Flavonoid biosynthesis	map	15	9	0.000127167768929279	0.00553179794842364
Stilbene, coumarine and lignin biosynthesis	map	10	6	0.0019891434536893 2	0.0576851601569903
Phenylalanine metabolism	map	14	6	0.0161784636552823	0.35188158450239
Alkaloid biosynthesis II	map	8	4	0.0277052903000527	0.477979365680773

Parameters

Amino acid

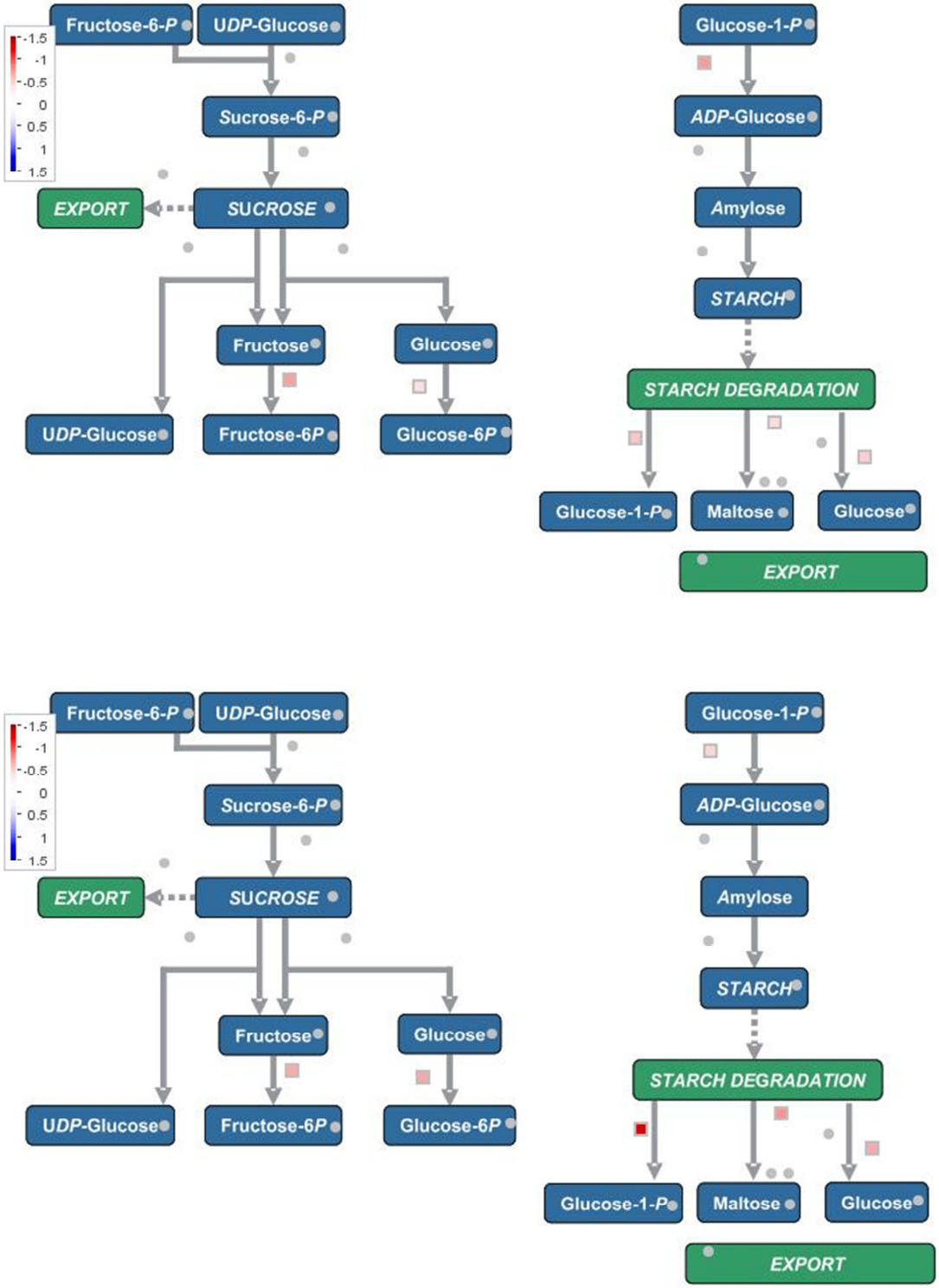
Genome Array:	Lotus Genome Array (Lotus japonicus)
Nb. of Identifiers submitted:	5671
EC assignment:	481 EC <-> 1526 identifiers view
Threshold:	0.1
Adjustment method:	fdr

Results

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted	P-value	Adjusted p-value
Porphyrin and chlorophyll metabolism	map	20	19	0.000302048786153847	0.0365479031246155
Carbon fixation	map	22	20	0.000908950408965812	0.0549914997424316
Glycolysis / Gluconeogenesis	map	27	23	0.00265085682886049	0.106917892097373
Starch and sucrose metabolism	map	31	25	0.00740517693686854	0.224006602340273
Glyoxylate and dicarboxylate metabolism	map	19	16	0.0163151565131383	0.394826787617947

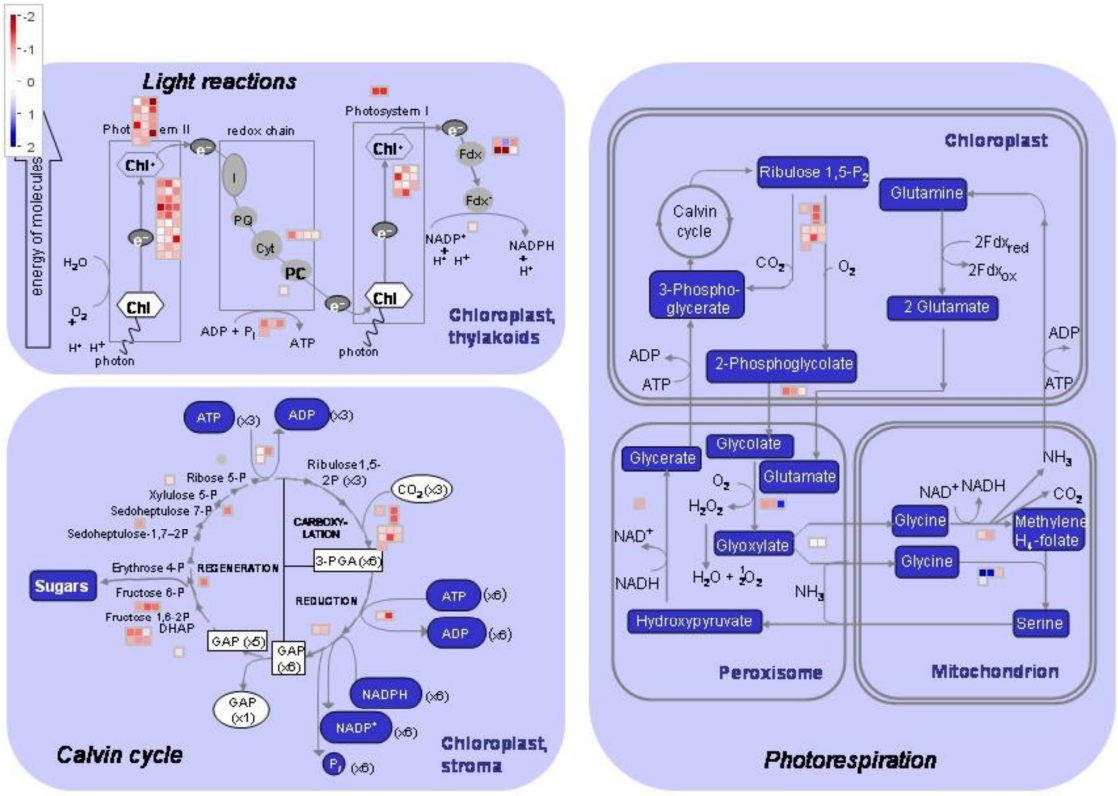
Supplemental Figure S7. Pathexpress analysis of overrepresented pathways. Significantly over-represented pathways ($P < 0.1$, FDR correction) among the probesets modulated by the transfer from high CO_2 to normal CO_2 conditions exclusively in WT plants (WT only), in both genotypes (Shared) and exclusively in the mutant (*Ljgln2-2* only) are highlighted in red.

Supplemental Figure S8:



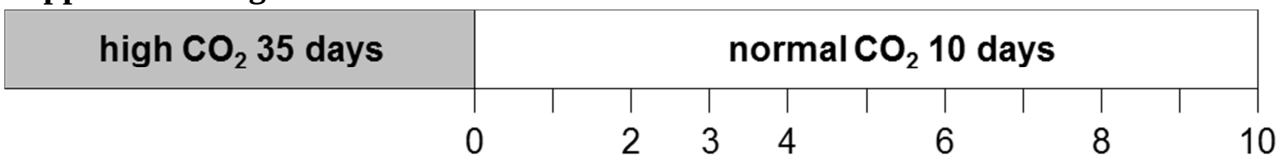
Supplemental Figure S8. MapMan metabolism overview of genes for starch and sucrose metabolism modulated by transfer from high CO_2 to normal CO_2 conditions both in WT and mutant plants. (a) Fold-change of the probesets in WT plants. (b) Fold-change of the same probesets as in (a) but in the mutant plants. The scale bar is shown in $\log_2$. Other details as previously described.

Supplemental Figure S9:



Supplemental Figure S9. MapMan overview of photosynthetic and photorespiratory genes modulated in *Ljgln2-2* by the transfer from high CO_2 to normal CO_2 conditions. The scale bar is shown in $\log_2$. Other details as described previously

Supplemental Figure S10:



Supplemental Figure S10. Experimental design used in this work. After 35 days of growth under high CO_2, WT and *Ljgln2-2* mutant plants were transferred to normal CO_2 conditions. Samples for RNA and metabolite extraction were taken at indicated time points. More details in materials and methods.

Supplemental Tables S1, S2, S3, S4 and **S5** are provided in the electronic version.

Publicación 2.

Alternative reassimilation of photorespiratory ammonium in *Lotus japonicus*.

Carmen M. Pérez-Delgado; Margarita García-Calderón; Antonio J. Márquez; Marco Betti

Unpublished.

ABSTRACT

The metabolic events associated to photorespiratory NH_4^+ accumulation were analyzed in a *Lotus japonicus* photorespiratory mutant lacking the plastidic isoform of glutamine synthetase. Measurements of gene expression, polypeptide levels, enzyme activity as well as metabolite profiling were carried out in leaf samples from WT and mutant plants after different periods of time under active photorespiratory conditions. The mutant plants accumulated high levels of NH_4^+ when photorespiration was active, followed by a sudden drop in the levels of this compound. Induction of genes encoding for cytosolic glutamine synthetase, glutamate dehydrogenase and asparagine synthetase were observed in the mutant in correspondence with the diminishment of NH_4^+ in leaves. Consistent with these results, the levels of the corresponding polypeptides and enzyme activities were increased exclusively in the mutant. In the case of asparagine synthetase it was not possible to determine enzyme activity and polypeptide content; however, an increase in Asn was observed in parallel with the induction of gene expression. A diminishment in Glu levels was observed, that may be explained by increased Gln synthesis and the induction of GDH activity, which is probably needed in order to produce carbon skeletons for NH_4^+ reassimilation. The data presented here represent a significant improvement in the study of nitrogen assimilation in *L. japonicus* and of the regulation of the key enzymes of this process; and suggest a role for Gln as a regulator of N gene expression.

INTRODUCTION

Nitrogen nutrition is one of the most crucial factors for plant productivity (Gutiérrez, 2012). Nitrate is the main form of nitrogen used by many crops, but most plants are also able to use ammonium, amino acids and urea (Andrews et al. 2013). Symbiotic N_2 fixation in the root nodule of legumes also provides important amount of reduced nitrogen to crops without the need of extensive fertilization (Udvardi and Poole, 2013). The assimilation of NO_3^- requires its prior reduction to NH_4^+ by the action of nitrate reductase and nitrite reductase (Masclaux-Daubresse et al. 2010). The NH_4^+ that has been taken up directly from the soil or produced by the reduction of NO_3^-, atmospheric N_2 in root nodules or urea hydrolysis, is then assimilated (generally in the roots) by the action of the glutamine synthetase (GS, EC 6.3.1.2.) / glutamate synthase (GOGAT, EC 1.4.7.1 and 1.4.1.14) cycle. In addition to primary nitrogen assimilation, other pathways are able to produce NH_4^+ like amino acid catabolism, phenylpropanoid biosynthesis, asparagine breakdown and photorespiration (Betti et al. 2012a). Photorespiration is probably the most important of these pathways due to the incredibly high flux and the different interconnections with other pathways showed by this route (Fernie et al. 2013). The large amounts of ammonium generated by the photorespiratory cycle are produced in the mitochondria as a result of the decarboxylation of two molecules of glycine to yield one molecule of serine by the action of glycine decarboxylase. NH_4^+ is then transferred to the chloroplast where is re-fixed by the GS/GOGAT cycle.

Plants have two types of GS isoenzymes: the cytosolic isoform of GS (or GS_1), that is generally encoded by a small gene family composed of 3-5 genes, and the plastidic GS isoform (or GS_2) that is generally encoded by a single gene, with the exception of *Medicago truncatula* where a second gene encoding for GS_2 has been described (Seabra et al. 2010). While GS_1 is the main enzyme involved in primary ammonium assimilation and recycling of the NH_4^+ generated during senescence and phenylpropanoid biosynthesis (Bernard and Habash 2009), GS_2 is involved in the reassimilation of the ammonium generated during photorespiration. The key role played by GS_2 in the reassimilation of photorespiratory ammonium was demonstrated by the isolation of mutant in barley that specifically lacked of this GS isoform (Wallsgrove et al. 1987). These mutants were conditionally lethal, since they grow well under a CO_2-enriched atmosphere (>0.2%), where photorespiration is suppressed, but showed stress symptoms and accumulated ammonium when transferred to normal CO_2 conditions (about 0.04%). Later on, the first photorespiratory mutants from legume plants were isolated in the model legume *Lotus japonicus* (Orea et al.

2002). These mutants were also specifically affected in the gene encoding for plastidic GS (Betti et al. 2006) and accumulated high levels of ammonium.

A recent work from our group made use of a *L. japonicus* plastidic GS mutant called *Ljgln2-2* in order to study the transcriptomic and metabolic consequences of the lack of plastidic GS both under high and normal CO_2 conditions (Pérez-Delgado et al. 2013). In a previous study we showed that the *L. japonicus Ljgln2-2* mutant, that lacks of the plastidic GS_2, accumulated high levels of NH_4^+ when transferred from high CO_2 conditions (suppressed photorespiration) to normal CO_2 (active photorespiration) (Pérez-Delgado et al. 2013). WT and mutant plants were grown under high CO_2 conditions for 35 days and then transferred to normal CO_2 conditions for different periods of time up to 10 days. Leaf samples were taken at different periods of time for both genotypes (Fig. 1). Interestingly, the ammonium levels in the mutant reached a maximum after 3 days under normal CO_2 conditions followed by a sudden decrease. A coordinate repression of the transcription of most photorespiratory genes was observed when the plants were shifted to normal CO_2 conditions, which may in part explain the drop in ammonium levels observed after 3 days. However, transcriptomic analysis indicated that important genes involved in N assimilation were modulated in the mutant under normal air conditions, including genes encoding for cytosolic GS_1 (Pérez-Delgado et al. 2013; Betti et al. 2014). This was part of a greater modulation of the transcriptome as well as of the metabolic profile of the mutant. A highly expressed gene encoding for cytosolic GS (*LjGln1.2*) was induced and this was paralleled by an increase in total GS activity. These preliminary results indicated that other enzymes besides plastidic GS_2 may be involved in the re-assimilation of photorespiratory ammonium in the *Ljgln2-2* mutant.

Primary and photorespiratory ammonium assimilation may take place by four different pathways (Potel et al. 2009): 1) Through the GS/GOGAT cycle previously described. 2) Glutamate dehydrogenase (GDH, EC 1.4.1.2) deaminates glutamate to 2-oxoglutarate and ammonium in order to replenish the TCA cycle of intermediates (Tercé-Lafourgue et al. 2013), but under stress conditions may also NH_4^+ in the reverse reaction, also called aminating activity (Skopelitis et al. 2006). 3) Through the action of asparagine synthetase (ASN, EC 6.3.5.4 and 6.3.1.1), which main reaction is the incorporation of the amino group of glutamine into aspartate to give asparagine, but can also use NH_4^+ as amino donor for Asn biosynthesis. 4) Finally, carbamoylphosphate synthetase (CPS, EC 6.3.4.16 and 6.3.5.5) produces carbamoylphosphate (CP) from HCO_3^-, ATP and either NH_4^+ or glutamine.

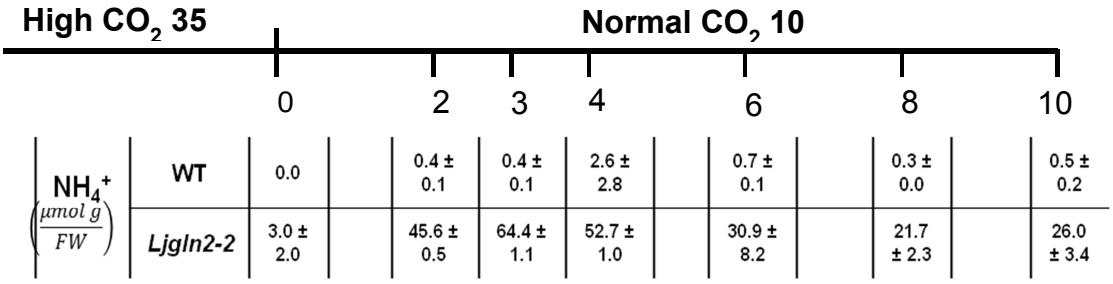


Fig. 1 Experimental design used in this work and ammonium content in WT and *Ljgln2-2* mutant leaves. WT and mutant plants were grown under high CO_2 conditions (0.7% v/v) and then transferred to normal CO_2 conditions (0.04% v/v). Samples for total NH_4^+ determination, as well as for RNA and metabolite extraction were taken at the indicated time points. Data are the mean ± SD of three independent biological replicates.

In this paper we present a complete study of all the metabolic reactions that may be involved in NH_4^+ detoxification in the *Ljgln2-2* photorespiratory mutant. For this, we carried out measurement of gene expression, polypeptide content, and enzyme activity as well as of the relative levels of the metabolites involved in the corresponding reactions. The data presented here demonstrate that cytosolic GS_1, GDH and ASN are induced in the mutant in correspondence to NH_4^+ accumulation, and suggest a role for these enzymes in NH_4^+ detoxification in *L. japonicus*. A model for the distribution of the NH_4^+ generated in the mutant under active photorespiratory conditions is also presented.

MATERIALS AND METHODS

Plant material and growth

Lotus japonicus (Regel) Larsen cv. Gifu was initially obtained from Professor Jens Stougaard (Aarhus University, Denmark) and then self-propagated at the University of Seville. WT and *Ljgln2-2* mutant plants were grown as described by Pérez-Delgado et al. (2013). For *Ljgln2-2*, the mutant progeny of two consecutive backcrosses into the WT background was used in this work. WT and mutant seeds were scarified and surface-sterilized, then germinated in 1% (w/v) agar in Petri dishes and transferred to pots using vermiculite as solid support. Five seedlings were planted in each pot and grown during 35 d in a growth chamber under 16 h : 8 h day : night, 20 : 18°C, with a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1} and a constant humidity of 70%. CO_2 was automatically injected to a final concentration of 0.7% (v/v) to allow for normal growth of the *Ljgln2-2* mutant in a photorespiration-suppressed atmosphere. Plants were watered with “Hornum” nutrient solution, containing

5 mM NH_4NO_3 and 3 mM KNO_3 (Handberg and Stougaard, 1992). After 35 days of growth under high CO_2 atmosphere, leaf tissue was harvested for each plant genotype, constituting the time zero point (photorespiration suppressed conditions). The plants were then transferred to normal air (0.04% CO_2 v/v) and each plant genotype was sampled at different time points (Fig. 1). The leaf tissue was flash-frozen in liquid N_2, grinded with a pestle in a mortar that was pre-cooled with liquid N_2 and the powder was stored at -80 ºC until use. Three independent biological replicates were harvested for each genotype and time point. A biological replicate consisted of tissue pooled from five plants grown in the same pot.

RNA extraction, cDNA synthesis and qRT-PCR

RNA extraction was carried out using the hot borate method as described by Sánchez et al. (2008). cDNA synthesis was carried out with the Superscript III reverse transcriptase exactly as described by Pérez-Delgado et al. (2013). qRT-PCR was carried out in a LightCycler 480 thermal cycler (Roche) using the SensiFAST SYBR No-ROX Kit (Bioline) also using the same conditions described by Pérez-Delgado et al. (2013). Expression levels were quantified by determining the C_T cycle of each reaction with the LightCycler 480 software version 1.5.0. Analysis of expression data was performed using the geometric mean of four housekeeping genes for normalization. The housekeeping genes were *LjGPI*-anchored protein (probeset: chr3.CM0047.42), *LjPp2A* (probeset: chr2.CM0310.22), *LjUbc10* (probeset: chr1.TM0487.4) and *LjUbq* (probeset: chr5.CM0956.27); these genes were selected amongst the most stably expressed genes in plants (Czechowski et al., 2005). A list of all the primer used for qRT-PCR is provided Table S1.

Preparation of crude extracts

Frozen leaf tissue was extracted with a pellet homogeniser in 5 ml / g FW of extraction buffer. The homogenated was centrifuged for 15 min at 15,000 g and 4 ºC and the supernatant constituted the crude extract. The extraction buffer for GS activity assay and immunoblot was prepared according to Orea et al., 2002. For GDH activity and immunoblot the extraction buffer was 100 mM Tris·HCl pH 8 with 1% of polyvinylpolypyrrolidone. The extraction buffer for CPS activity assay was prepared according to Pierson et al. 1980.

Western Blot

Western blots from denaturing PAGE were performed using the ECL Western blotting system (GE Healthcare) according to the manufacturer instructions. SDS-PAGE was performed according to the method of Laemmli (1970) using a Mini-Protean Tetra Cell system (Bio-Rad). An acrylamide concentration of 12%, 10% and 12.5 (w/v) was used for the SDS-PAGE running gels used for immunodetection of GS, GDH and ASN respectively. Stacking gels were always prepared at 4% (w/v) acrylamide. 10 μg of total protein from leaf crude extracts were loaded in each well of the gels. Protein concentration was quantified using the Bradford protein assay (Bio-Rad). The polypeptide molecular weights were estimated using the broad range molecular weight standards (Bio-Rad).

Anti-GS primary antibodies were raised in rabbit immunised with the recombinant homopolymeric α GS from *Phaseolus vulgaris* purified by metal-affinity chromatography (Betti et al. 2002) and used at 1:1,000 dilution. Anti-GDH primary antibodies (a gift from K.A. Roubelakis-Angelakis, University of Crete) were raised in rabbit using purified NADH-GDH from grapevine (Loulakakis and Roubelakis-Angelakis 1990). Anti-ASN antibodies (a gift from FM Cánovas and RA Cañas, University of Málaga) were raised in rabbit using purified ASN from Scots pine (*Pynus sylvestris L.*) as described by Cañas et al. (2006). Anti-rabbit secondary antibodies labelled with peroxidase were obtained from GE Healthcare.

Detection of peroxidase signal in the membranes was carried out with a quimioluminescence detection system (Fujifilm LAS 3000 mini, Fujifilm). Relative quantification of the polypeptide levels was carried out for three different biological replicates for each time point and genotype. Densitometry measurements were performed with the ImageJ software (http://rsb.info.nih.gov/ij/).

Enzyme activity assays

GS enzyme activity was determined by measuring ATP hydrolysis as described by Márquez et al. (2005). GDH aminating and deaminating activities were measured as described by Loulakakis and Roubelakis-Angelakis, 1991, except that it was used 0.1 mM of NADH/NADPH in the aminating reaction.

CPS enzyme activity was determined according to Doremus (1986). Assays for ASN enzyme activity were carried out according to Sheng et al. (1993).

Metabolite profiling analysis

Metabolite profiling analysis was carried out using the dataset generated by Pérez-Delgado et al. (2013). Statistical differences between metabolite levels were assessed with two-way ANOVA at $P < 0.001$ using the Multiexperiment Viewer version 3.1 (Saeed et al. 2006) and the factors "genotype" and "time under normal CO_2". Only metabolites that showed significant differences according to this analysis were considered. The relative metabolite levels presented in this paper are the direct normalized responses of metabolite pool measures, that is, mass detector signals in arbitrary units normalized to internal standard and sample fresh weigh.

Ammonia determination was carried out according to the method of Solorzano (1969) with some modifications as described by Orea et al. (2002). CP content in crude extracts was determined with the same colorimetric method used for CPSase activity but using undialyzed extracts.

RESULTS

Ammonium accumulation and induction of cytosolic GS_1 in the Ljgln2-2 mutant

The drop in ammonium levels observed after the maximum at day 3 in the mutant (Fig. 1) suggested a possible regulation of the photorespiratory cycle and/or induction of alternative routes for secondary ammonium assimilation in this genotype. Preliminary results indicated that a gene encoding for a cytosolic GS_1 isoform was induced in the *Ljgln2-2* mutant after the transfer to normal CO_2 conditions, and this was paralleled by an increase in total GS activity (Pérez-Delgado et al. 2013). In order to study more in detail this response, all the sequence information available on *L. japonicus* cytosolic GS_1 genes was obtained from the available databases. A total of five unique gene sequences encoding for cytosolic GS_1 were found (not shown). Gene specific oligonucleotides were used in order to determine transcription levels in WT and mutant plants by qRT-PCR at the different time points considered. *LjGln1.2* was the most expressed GS_1 gene in leaves and was induced almost two-folds in the mutant, with a maximum in transcript levels at day 3 followed by a slow decrease (Fig. 2a). Interestingly,

LjGln1.2 transcription over time followed a similar trend in the WT plants, although in this genotype the transcript levels of *LjGln1.2* were always about half of the mutant ones at all the time points. Both *LjGln1.1* and *LjGln1.3* showed a sudden decrease in transcript levels in both genotypes, followed by a recover in transcript levels at days 6-8 exclusively in the mutant. Finally, *LjGln1.4* and *LjGln1.5* were expressed at very low levels and were repressed in both genotypes after the shift to normal CO_2 conditions. Western Blot analysis was carried out using Anti-GS antibodies that recognize both cytosolic and plastidic GS. While GS_1 and GS_2 could be separated according to their different molecular weights (Betti et al. 2006), it was not possible to distinguish between the different gene products of the five cytosolic GS_1 genes since their molecular weights are very similar. In agreement with the gene expression data, cytosolic GS_1 polypeptide levels were increased in the mutant during the time-course of the experiment. The maximum abundance of GS_1 was after 8 days under normal CO_2 conditions, were the polypeptide levels were about 4 times higher than in the WT (Fig. 2b). Neither GS_1 nor GS_2 polypeptide levels showed any significant change in the WT compared to high CO_2 conditions. Total GS enzyme activity was also increased exclusively in the *Ljgln2-2* mutant, and was in good agreement with GS_1 polypeptide levels, indicating that the increased amount of GS_1 was represented by active enzyme (Fig. 2c). Glutamine levels were also increased in the mutant, with a maximum at day 4, just after the drop in NH_4^+ levels. These data suggest a role for cytosolic GS_1 as an alternative route for photorespiratory NH_4^+ detoxification. Interestingly, in the WT a drop in Gln levels to about half of the initial values was observed throughout the experiment, even if GS polypeptide levels and enzyme activity did not show significant changes. On the other hand, Glu relative levels were decreased about two-folds in both genotypes as a consequence of the transfer to normal CO_2 conditions.

Induction of GDH in the Ljgln2-2 mutant

Three different gene sequences encoding for NAD(H)-GDH (*LjGDH1*, *LjGDH2* and *LjGDH3*) as well as a fourth gene encoding for a putative NADP(H)-dependent GDH enzyme (*LjGDH4*) were found in the *L. japonicus* genome. Two of the three genes encoding for NAD(H)-GDH in the mutant were induced after 4 days under normal CO_2 conditions (*LjGDH2* and *LjGDH3*, Fig. 3a). On the other hand, the most expressed GDH gene in leaves (*LjGDH1*) was not significantly modulated in the mutant and was repressed about three fold in the WT genotype. *LjGDH4* transcript levels showed in both genotypes a sudden drop at day 2 followed by a recuperation over time in both genotypes, with a trend that was almost opposite to NH_4^+ levels. Two main immunoreactive bands

were detected by Western blot analysis, one compatible with the size of the NAD(H)-GDH polypeptides (about 44,700 Da for all the three possible isoforms) and one with higher molecular weight that was compatible with the size of the putative NADP(H)-GDH polypeptide (about 71,000 Da).

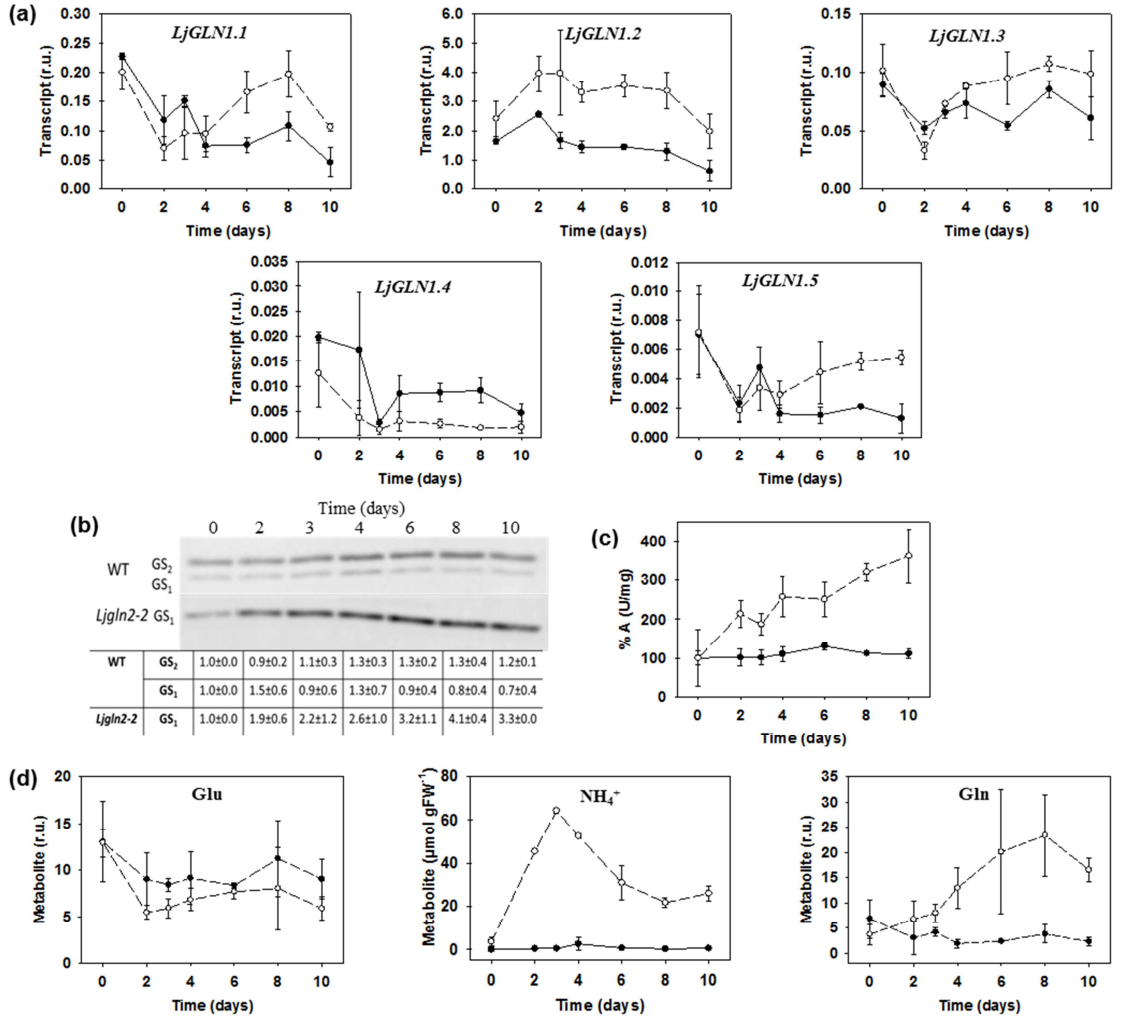


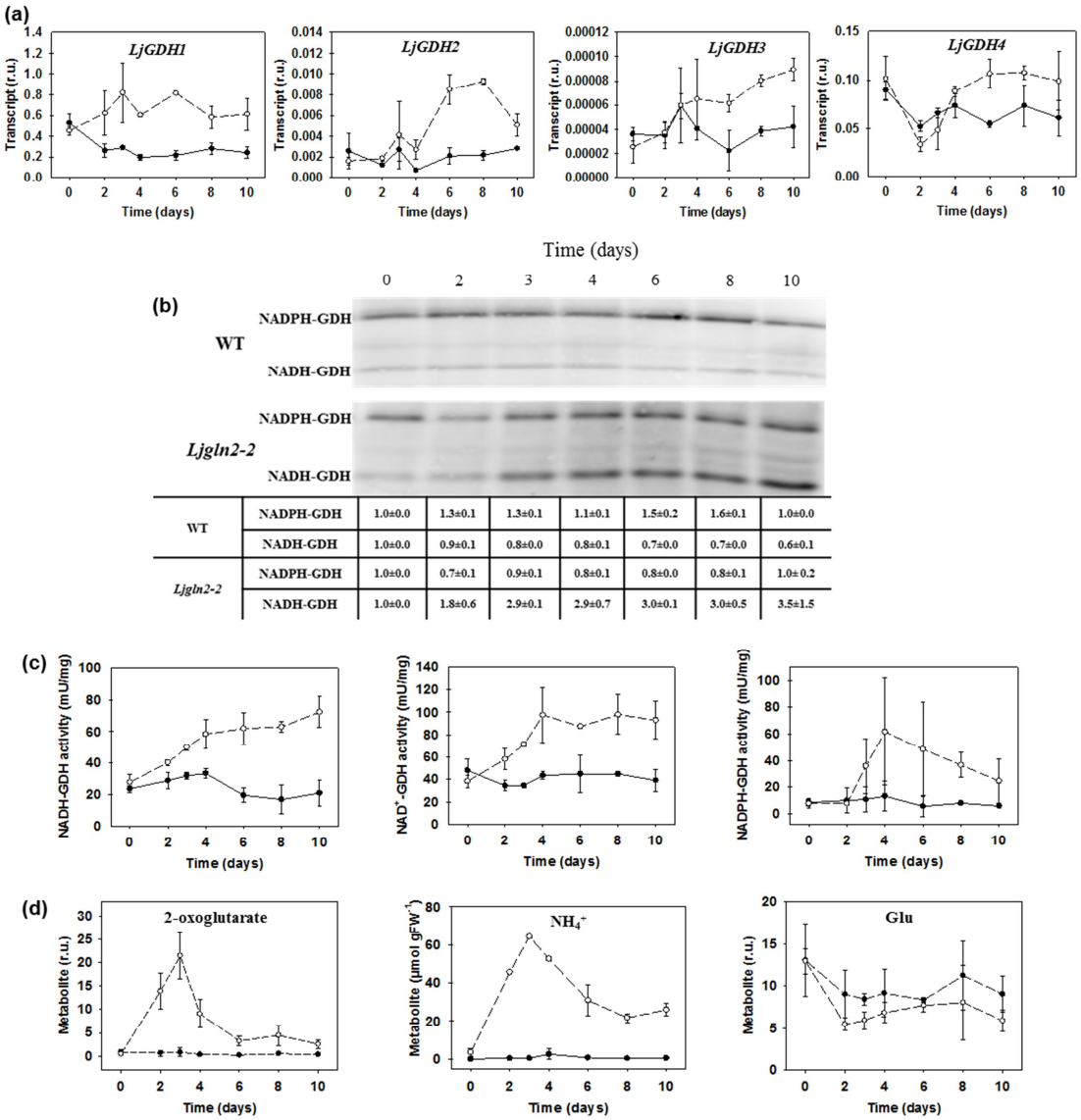
Fig. 2 Regulation of GS in WT and *Ljgln2-2* mutant plants after the transfer to active photorespiratory conditions. **(a)** Expression of the genes for cytosolic GS_1 in WT and mutant plants. Transcript levels were quantified by qRT-PCR in WT (black dots, black line) and mutant (white dots, dashed line) leaves at the same time points than in Fig. 1. Relative transcript levels are reported for each genotype compared to the housekeeping genes. **(b)** Immunodetection of cytosolic GS_1 and plastidic GS_2 in leaf crude extracts from WT and mutant plants. 10 µg of total protein were loaded on each lane. The position of the molecular weight markers is indicated. The table reports the densitometric quantification of the GS_1 and GS_2 bands. Data are the mean ± SD of three independent biological replicates. The blot shown in the Figure is only one of the different biological and technical replicates carried out. **(c)** The GS specific activity was determined in crude extracts from leaves of WT (black dots, solid line) and *Ljgln2-2* (white dots, dashed line). GS activity is reported as specific activity (U/mg of total protein). Data are the mean ± S.D. of three independent biological replicates. **(d)** Relative amount of substrates and products of the GS reaction. WT: black dots, solid line, *Ljgln2-2* mutant: white dots, dashed line. Metabolite levels are reported as relative units (r.u.) in the case of Glu and Gln or µmol·g FW in the case of NH_4^+.

Quantification of the immunoblots indicated an increase of more than three times in NAD(H)-GDH polypeptide levels in the mutant, while in the WT they were almost unchanged (Fig. 3b). On the other hand, NAPD(H)-GDH polypeptide levels were slightly increased in the WT but were not significantly changed in the mutant. GDH enzyme activity was determined in both directions (aminating and deaminating activity). Both activity assays indicated an increase of about 3-4 times in GDH enzyme activity exclusively in the mutant, in very good agreement with the polypeptide levels. NADPH-GDH enzyme activity was also induced in the mutant, although with more variation between biological replicates compared to the NAD(H)-GDH activity (Fig. 3c). Curiously, the NADP-dependent deaminating activity was undetectable in WT and mutant extracts (not shown). Analysis of the metabolite levels of the substrates and products of GDH reaction showed that the levels of 2-oxoglutarate were increased in the mutant, with a trend over time very similar to the ammonium one (Fig. 3d). As described before, Glu leaf content was decreased about two-folds in both genotypes after the shift to active photorespiratory conditions.

Transcriptional modulation of ASN genes

Two genes were initially described as encoding for ASN in *L. japonicus* (Waterhouse et al. 1996): *LjAS1* and *LjAS2*. Here we will call these two genes *LjASN1* and *LjASN2* respectively according to the current nomenclature for asparagine synthetase genes. However, mining of the available database information led to the identification of a third *ASN* gene, that we will call *LjASN3*.

Information about the role of ASN in plants came mainly from measurements of gene expression and metabolite levels. This is because of the difficulty of determine ASN polypeptide levels and enzyme activity due to the rapid turnover of the enzyme, to the presence of asparaginase activity and of ASN inhibitors in the extracts (Azevedo et al. 2006). This was also the case of *L. japonicus* leaves extracts since it was not possible to determine ASN enzyme activity and no inmunoreactive bands were found using Anti-ASN primary antibodies (results nor shown). qRT-PCR analysis showed induction of the *LjASN2* gene, the less expressed of the three ASN genes, exclusively in the mutant (Fig. 4a). *LjASN1*, the most expressed one, did not show a clear trend over the time-course of the experiment but was always more expressed in the WT than in the mutant, even at time zero (high CO_2 conditions) were most genes showed similar expression.



		0	2	3	4	6	8	10
WT	NADPH-GDH	1.0±0.0	1.3±0.1	1.3±0.1	1.1±0.1	1.5±0.2	1.6±0.1	1.0±0.0
	NADH-GDH	1.0±0.0	0.9±0.1	0.8±0.0	0.8±0.1	0.7±0.0	0.7±0.0	0.6±0.1
Ljgln2-2	NADPH-GDH	1.0±0.0	0.7±0.1	0.9±0.1	0.8±0.1	0.8±0.0	0.8±0.1	1.0± 0.2
	NADH-GDH	1.0±0.0	1.8±0.6	2.9±0.1	2.9±0.7	3.0±0.1	3.0±0.5	3.5±1.5

Fig. 3 Regulation of GDH in WT and *Ljgln2-2* mutant plants after the transfer to active photorespiratory conditions. **(a)** Expression of the genes for NAD(H)-GDH (*LjGDH1*, *LjGDH2* and *LjGDH3*) and NADP(H)-GDH (*LjGDH4*) in WT and mutant plants. Transcript levels were quantified by qRT-PCR in WT (black dots, black line) and mutant (white dots, dashed line) leaves at the same time points than in Fig. 1. Relative transcript levels are reported for each genotype compared to the housekeeping genes. **(b)** Immunodetection of NAD(H)-GDH and NADP(H)-GDH in leaf crude extracts from WT and mutant plants. 10 µg of total protein were loaded on each lane. The position of the molecular weight markers is indicated. The table reports the densitometric quantification of the NAD(H)-GDH and NADP(H)-GDH bands. Data are the mean ± SD of three independent biological replicates. The blot shown in the Figure is only one of the different biological and technical replicates carried out. **(c)** The GDH specific activity was determined in crude extracts from leaves of WT (black dots, solid line) and *Ljgln2-2* (white dots, dashed line). GDH activity is reported as specific activity (mU/mg of total protein). Data are the mean ± S.D. of three independent biological replicates. **(d)** Relative amount of substrates and products of the GDH reaction. WT: black dots, solid line, *Ljgln2-2* mutant: white dots, dashed line. Metabolite levels are reported as relative units (r.u.) in the case of 2-oxoglutarate and Glu or µmol·g FW in the case of NH_4^+.

A slight but significant accumulation of Asn was observed in the *LjGln2-2* mutant under active photorespiratory conditions (Fig. 4b). On the other hand, Asp levels were always higher in the WT plants than in the mutant, showing a trend quite similar to the transcript levels of *LjASN1*.

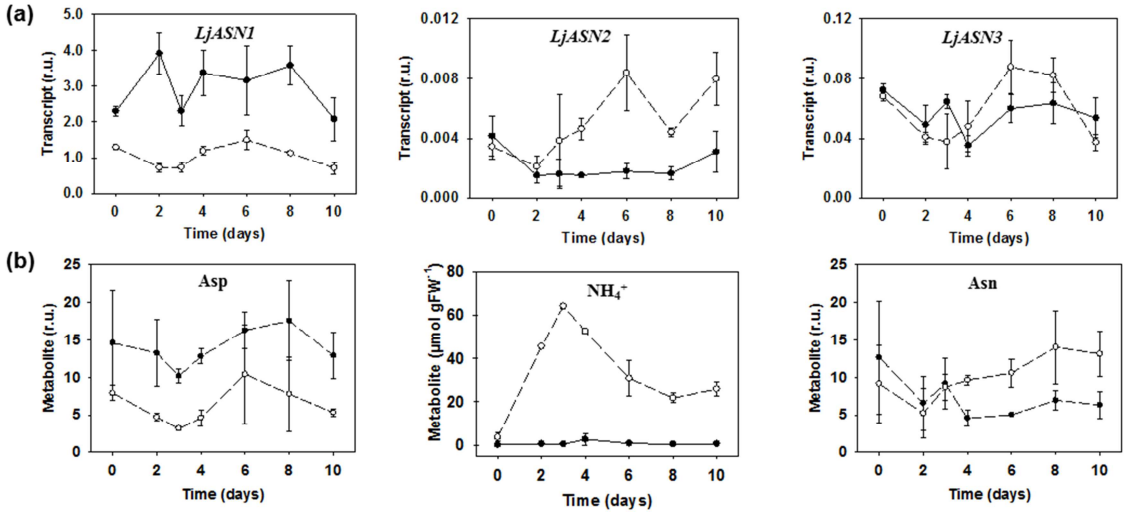


Fig. 4 Regulation of ASN in WT and *Ljgln2-2* mutant plants after the transfer to active photorespiratory conditions. **(a)** Expression of the genes encoding for ASN in WT and mutant plants. Transcript levels were quantified by qRT-PCR in WT (black dots, black line) and mutant (white dots, dashed line) leaves at the same time points than in Fig. 1. Relative transcript levels are reported for each genotype compared to the housekeeping genes. **(b)** Relative amount of substrates and products of the NH_4^+-dependent ASN reaction. WT: black dots, solid line, *Ljgln2-2* mutant: white dots, dashed line. Metabolite levels are reported as relative units (r.u.) in the case of Asp and Asn or µmol·g FW in the case of NH_4^+.

CPS gene expression and CP content in WT and Ljgln2-2

Plant CPS is similar to the bacterial enzyme and is formed by a large and a small subunit (Slocum, 2005). Most plants have two genes encoding for the small and large CPS subunits respectively, that in Arabidopsis are called *carA* and *carB* (Potel et al. 2009). In *L. japonicus*, two genes for CPS were found: *LjcarA* and *LjcarB*, which encode for the small and large CPS subunits respectively. The two genes showed similar levels of expression in *L. japonicus* leaves (Fig. 5a). Only *LjcarB* was modulated by the transfer to active photorespiratory conditions, with a repression of about three times in expression after 4 days followed by a recover of transcript levels in both genotpyes. CP metabolite content was slightly increased in *Ljgln2-2* over time, while in the WT genotype was increased more that two-folds with a peak at day 2 followed by a sudden decrease in metabolite levels (Fig. 5b).

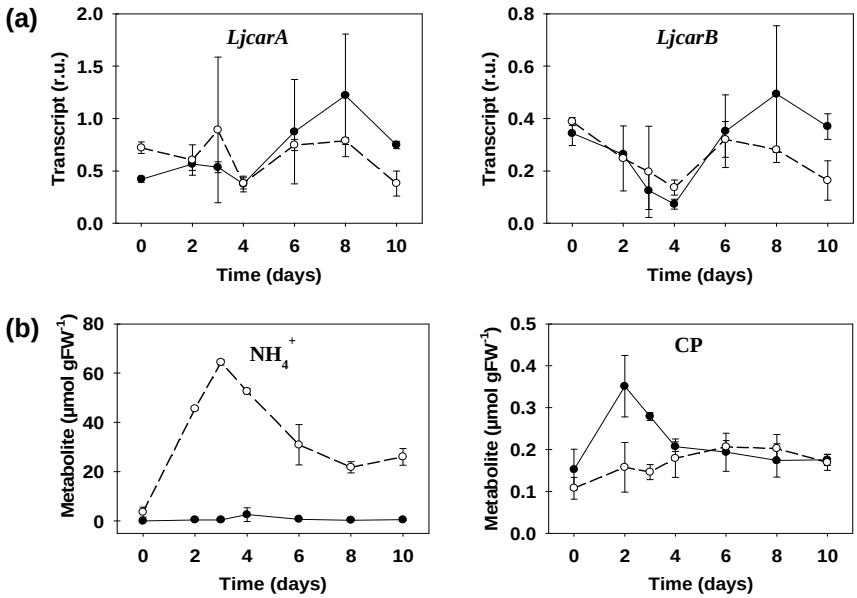


Fig. 5 Regulation of CPS in WT and *Ljgln2-2* mutant plants after the transfer to active photorespiratory conditions. **(a)** Expression of the genes encoding for the CPS small subunit (*LjcarA*) and large subumit (*LjcarB*) in WT and mutant plants. Transcript levels were quantified by qRT-PCR in WT (black dots, black line) and mutant (white dots, dashed line) leaves at the same time points than in Fig. 1. Relative transcript levels are reported for each genotype compared to the housekeeping genes. **(b)** Relative amount of substrates and products of the NH_4^+-dependent ASN reaction. WT: black dots, solid line, *Ljgln2-2* mutant: white dots, dashed line. Metabolite levels are reported as µmol·g FW.

DISCUSSION

Ljgln2-2 mutant plants accumulated high levels of NH_4^+ under active photorespiratory conditions followed by a sudden decrease in the content of this compound. This was suggestive of a regulation of photorespiratory metabolism and/or of enzymes involved in secondary NH_4^+ assimilation. Since the transcriptional regulation of photorespiratory and photosynthetic genes was already described (Pérez-Delgado et al. 2013), in this paper we focused on the enzymes that may detoxify photorespiratory NH_4^+ in the *Ljgln2-2* mutant. In the following sections we discuss the regulation of the different enzymes considered.

Glutamine synthetase

Cytosolic GS_1 is encoded in plants by a small gene family that is generally formed by 3-5 members (Swarbreck et al. 2011). In the *L. japonicus* genome we found five different unique sequences encoding for cytosolic GS_1. The corresponding genes showed different levels of expression as well as different responses to the transfer from high to normal CO_2 conditions in the leaves of WT

and *Ljgln2-2* plants. Importantly, the most expressed *GLN1* gene in leaves was induced in the mutant in correspondence with the peak in NH_4^+ levels, and this was paralleled by an increase in GS_1 polypeptide levels and enzyme activity. While the enzyme involved in the re-assimilation of photorespiratory NH_4^+ is plastidic GS_2, the data presented here suggest that in the presence of high levels of NH_4^+ like the observed in the mutant, cytosolic GS_1 may help in the detoxification of this compound. In fact, previous reports have shown that overexpression of cytosolic GS_1 in leaf mesophyll cells of tobacco resulted in a reduction of free ammonium levels in leaves under active photorespiratory conditions (Oliveira et al. 2002). Moreover, cytosolic GS_1, as well as ASN and GDH are induced in old senescing leaves in order to reassimilate the NH_4^+ generated by protein degradation (Masclaux-Daubresse et al. 2010). Even if the establishment of leaf senescence in the latter time points of our experiment is possible, the data presented here for GS_1 (as well as for ASN and GDH) indicate that the induction of some of these genes/enzyme activities is probably an early stress response aimed to NH_4^+ detoxification. In fact, induction of GS and GDH enzyme activity, as well as ASN gene expression, were observed after 2-3 days under active photorespiratory conditions, were the leaves did not show any apparent symptoms of stress.

There are evidences that indicate that NH_4^+ itself regulates the transcription of *GLN* genes (Masclaux-Daubresse et al. 2010; Hirel et al. 1987). However, previous reports also suggested a role for both NH_4^+ and Gln in the signalling route that leads to modulation of gene expression in order to re-assimilate photorespiratory NH_4^+ (Ferrario-Mery et al. 2002). Since both Gln and NH_4^+ levels are increased in the mutant under active photorespiratory conditions, both molecules may play a role in the induction of GS_1 gene expression. However, this does not exclude that other metabolites may participate in the regulation of *GLN1* gene transcription.

It has to be taken into consideration that the different products of the cytosolic GS_1 gene family are generally located in different cells of the vascular tissue, while plastidic GS_2 is normally located in the photosynthetic tissue, where NH_4^+ production by the photorespiratory cycle also occurs (Seabra et al. 2013; Bernard and Habash, 2010). If this is the case in *L. japonicus*, then some transport or diffusion mechanism between photosynthetic and vascular tissues should function in order to shift the accumulated NH_4^+ in the mutant to the tissues were cytosolic GS_1 is present. Further studies carried out with a higher cellular resolution will be needed in order to get more insight into the intercellular traffic of N metabolites in this model legume.

Glutamate dehydrogenase

According to the available sequence information, three genes encoding for NAD(H)-GDH and one for a putative NADP(H)-GDH isoforms are present in the *L. japonicus* genome. This is in agreement with recent studies in Arabidopsis, where the same number of genes encoding for NAD(H)-GDH and for the putative NADP(H)-GDH has been described (Fontaine et al. 2012; Fontaine et al. 2013). Glutamate dehydrogenase can catalyze *in vitro* both the NADH-dependent incorporation of ammonium into 2-oxoglutarate to give glutamate (aminating reaction) or the NAD-dependent deamination of glutamate to give 2-oxoglutarate and ammonium (deaminating reaction). However, recent evidences strongly suggest that the enzyme works in the direction of glutamate deamination in order to provide 2-oxoglutarate to the Krebs cycle and to control Glu homeostasis (Fontaine et al. 2012; Labboun et al. 2009). Given the high levels of NH_4^+ present in leaves of the *Ljgln2-2* mutant, it may be possible that an increased deamination of Glu by GDH is needed in order to provide carbon skeletons for its re-assimilation. The metabolite profiles presented in this work for the *Ljgln2-2* mutant support this hypothesis, since Glu levels drop after the shift to active photorespiratory conditions. An activation of the anaplerotic reactions of the Krebs cycle in order to provide carbon skeletons was also observed in a tobacco Fd-GOGAT mutant under active photorespiratory conditions and NH_4^+ accumulation (Ferrario-Mery et al. 2002). The authors also observed induction of GDH activity in the mutant under active photorespiratory conditions (Ferrario-Mery et al. 2002).

In a study carried out in tobacco, the expression of *GDH* genes was controlled by both C and N metabolites (Masclaux-Daubresse et al. 2005). Pro and Gln were major inducer of tobacco *GDH*, while sucrose repressed *GDH* expression (Masclaux-Daubresse et al. 2005). While Pro levels diminished in WT and *Ljgln2-2* as a consequence of the transfer to active photorespiratory conditions (Pérez-Delgado et al. 2013), Gln leaf content was increased in the mutant and diminished in the WT (Fig. 2). This suggests that Gln may be a good candidate for *GDH* gene regulation. In particular, the expression of the *LjGDH1* is in very good agreement with this hypothesis, since it is induced in the mutant and repressed in the WT (Fig. 3).

A gene sequence encoding for a putative NADP(H)-GDH was detected in the *L. japonicus* genome. In *Arabidopsis*, only very low of NADP-dependent enzyme activity were detected after PAGE in-gel GDH activity staining of root, leaves and stem extracts. (Fontaine et al. 2013). Moreover, the activity bands

observed coincided with the NAD-GDH ones, suggesting that NADP(H)-dependent GDH is not expressed as an active protein (Fontaine et al. 2013). In this work we detected quite high levels of NADPH-dependent activity in the mutant under active photorespiratory conditions. Interestingly, when the NADP-dependent activity was assayed, no detectable enzyme activity was found, which is quite in agreement with the data of Fontaine et al. (2013) that assayed only the NADP-dependent GDH activity and not de NADPH-dependent one. The data presented here suggest that NADP(H)-GDH may be expressed as an active protein in *L. japonicus*, but that the corresponding enzyme activity is high only under particular situations, like the one present in the *Ljgln2-2* under active photorespiration.

Asparagine synthetase

Although the main amino donor for Asn biosynthesis in the reaction catalyzed by asparagine synthetase is Gln, this enzyme can also use NH_4^+ if the concentration of this molecule is high enough (Lea et al. 2007). Even if the importance of this ammonium-dependent pathway *in vivo* has still not been clarified, there are studies that suggest a role for ASN in NH_4^+ detoxification under stress conditions (Wong et al. 2004). Moreover, a study carried out in tobacco demonstrated that $^{15}NH_4^+$ is incorporated into Asn by the action of ASN (Masclaux-Daubresse et al. 2006).

All plants contain a small *ASN* gene family composed of 2-3 members (Gaufichon et al. 2010). Arabidopsis for example has, like *L. japonicus*, three *ASN* genes: *ASN1*, *ASN2* and *ASN3* (Gaufichon et al. 2013). Sequence analysis showed that *LjASN1* is similar to Arabidopsis *ASN1*, while *LjASN2* and *LjASN3* share more similarity with Arabidopsis *ASN2* and *ASN3* respectively. Phylogenetic analysis indicates that *Arabidopsis ASN1* clusters in the dicot-subclass I of asparagine synthetases, while *ASN2* and *ASN3* cluster separately in the dicot-subclass II (Gaufichon et al. 2010). The *ASN* genes belonging to the Class I show a different regulation from Class II *ASN*, suggesting a different role for the two classes of ASN (Herrera-Rodríguez et al. 2004). Of the three genes encoding for ASN found in the *L. japonicus* genome, two of them showed differential expression between WT and mutant (*LjASN1* and *LjASN2*), while *LjASN3* was modulated by the transfer to normal CO_2 atmosphere but did not show different expression between the two genotypes (Fig. 4). LjASN1 was the most expressed in leaves, confirming previous reports (Waterhouse et al. 1996). Only *LjASN2* was induced in the mutant after the transfer to normal CO_2

conditions. Considering that the trend in Asn over time were very similar to the trend in expression level of *LjASN2* in both genotypes (Fig. 5 a,b), these data suggest that *LjASN2* may be responsible for the Asn levels in *L. japonicus*. It has to be taken into consideration that plants may also produce Asn through β-cyanoalanine hydrolysis and transamination of 2-oxosuccinamic acid, however these reactions are probably of a lower magnitude compared to the one catalyzed by ASN (Gaufichon et al. 2010). The induction of *LjASN2* is also in agreement with the essential role of its Arabidopsis homolog *ASN2* in N assimilation, distribution and remobilization within the plant (Gaufichon et al. 2013). Moreover, Arabidopsis *ASN2* plays a role in NH_4^+ recycling under stress (Lam et al. 2003). This is consistent with the induction of *LjASN2* exclusively in the mutant and suggests a role for this gene in NH_4^+ detoxification. On the other hand, it is interesting to notice that *LjASN1* is much more expressed in the WT than in the mutant, even under control conditions (suppressed photorespiration, Fig. 5a, time 0). Further studies will be needed in order to get more insight into the function of each of the three *LjASN* genes, including the effects of light, carbohydrates and N supply.

Carbamoylphosphate synthetase

Carbamoylphosphate synthase provides CP for both pyrimidine synthesis pathway (Slocum, 2005) and for arginine biosynthesis (Mollá-Morales et al. 2010). However, recent studies have shown that CPS can also play a role in NH_4^+ assimilation (Potel et al. 2009). In fact, CP production by CPS is used for the formation of citrulline, which is a precursor for arginine biosynthesis. This pathway may help to detoxify excess NH_4^+ by means of Arg production (Potel et al. 2009). For this reason, we decided to study if CPS may be modulated by active photorespiratory conditions in *L. japonicus* WT and mutant plants.

In the *L. japonicus* genome, two genes encoding for the small and large subunits of CPS were detected *(LjcarA* and *LjcarB* respectively). This is similar to the situation observed in most plants, with the exception of the model legume *Medicago truncatula*, where two different genes encode for the small CPS subunit (Brady et al. 2010). In both genotypes only the *LjCarB* gene was modulated by the transfer to active photorespiratory conditions, with a drop in transcript levels followed by a recover. qRT-PCR studies carried out in Arabidopsis showed that exogenous NH_4^+ lowered the expression of the *carB* but not of *carA* (Potel et al. 2009). This data suggest that CPS expression may be regulated by NH_4^+. It has to be pointed out that in WT plants, even in the

presence of normal levels of plastidic GS_2 activity, a slight but significant increase of leaf NH_4^+ content was observed after the transfer to normal CO_2 conditions (Fig. 1, day 4). These levels of NH_4^+ should then be enough in order to trigger the repression of *LjcarB* in both genotypes. Interestingly, CP metabolite levels were increased about two times exclusively in the WT genotype. This suggests that the CPS enzyme may be regulated at the post-transcriptional or post-translational levels in *L. japonicus*. Plant CPS is an allosteric enzyme, which activity is inhibited by UMP and activated by ornithine and PRPP (Moffatt and Ashihara, 2002). Since it was not possible to measure CPS enzyme activity in *L. japonicus* leaf extracts, this hypothesis on CPS regulation in *L. japonicus* remains for the moment speculative.

Alternative assimilation of NH_4^+ in the *Ljgln2-2* mutant

In this work we have considered the different enzyme activities and metabolic pathways that may be involved in the detoxification of photorespiratory NH_4^+ in the *Ljgln2-2* mutant that lacks of plastidic GS_2, the enzyme that is normally re-assimilating the ammonium released by the photorespiratory cycle. Besides of NH_4^+ itself, the central molecule in this metabolic puzzle is without any doubt glutamate (Fig. 6). Glu plays a central role in plant amino acid metabolism since it is responsible both of the assimilation of NH_4^+ and of the transfer of its α-amino group to all other amino acids. Glu levels diminished in the *LjGln2-2* mutant (but also in the WT) after the transfer to active photorespiratory conditions. This is interesting considering that Glu levels are remarkably stable in plants (Forde and Lea, 2007). The induction of cytosolic GS_1 activity to produce Gln may explain in part this drain of the Glu pool. On the other hand, GDH activity was induced exclusively in the mutant. This would lead to increased deamination of Glu in order to produce 2-oxoglutarate to sustain the GS/GOGAT cycle and to generate carbon skeletons for NH_4^+ assimilation. On the other hand, *Ljgln2-2* accumulated Thr, Ile and Lys, all amino acids that have Asp as their precursor (Azevedo et al., 2006). The accumulation of these amino acids should drain the Asp pool, demanding further Glu for the transamination of oxaloacetate for Asp biosynthesis. The scheme that can be generated from the data presented in this paper indicates that, in absence of plastidic GS_2, the excess of NH_4^+ present in the mutant is probably detoxified via an increased flux of the reactions involving Glu that leads to the accumulation of several amino acids. It has to be pointed out that the steady states of metabolites, and not the fluxes towards the relative metabolic pathways, were measured in this work. Nevertheless, the results obtained represent a significant improvement in the

study of nitrogen assimilation in *L. japonicus* and of the regulation of the key enzymes of this process. Finally, our results are compatible with a role for Gln as a regulator of N gene expression.

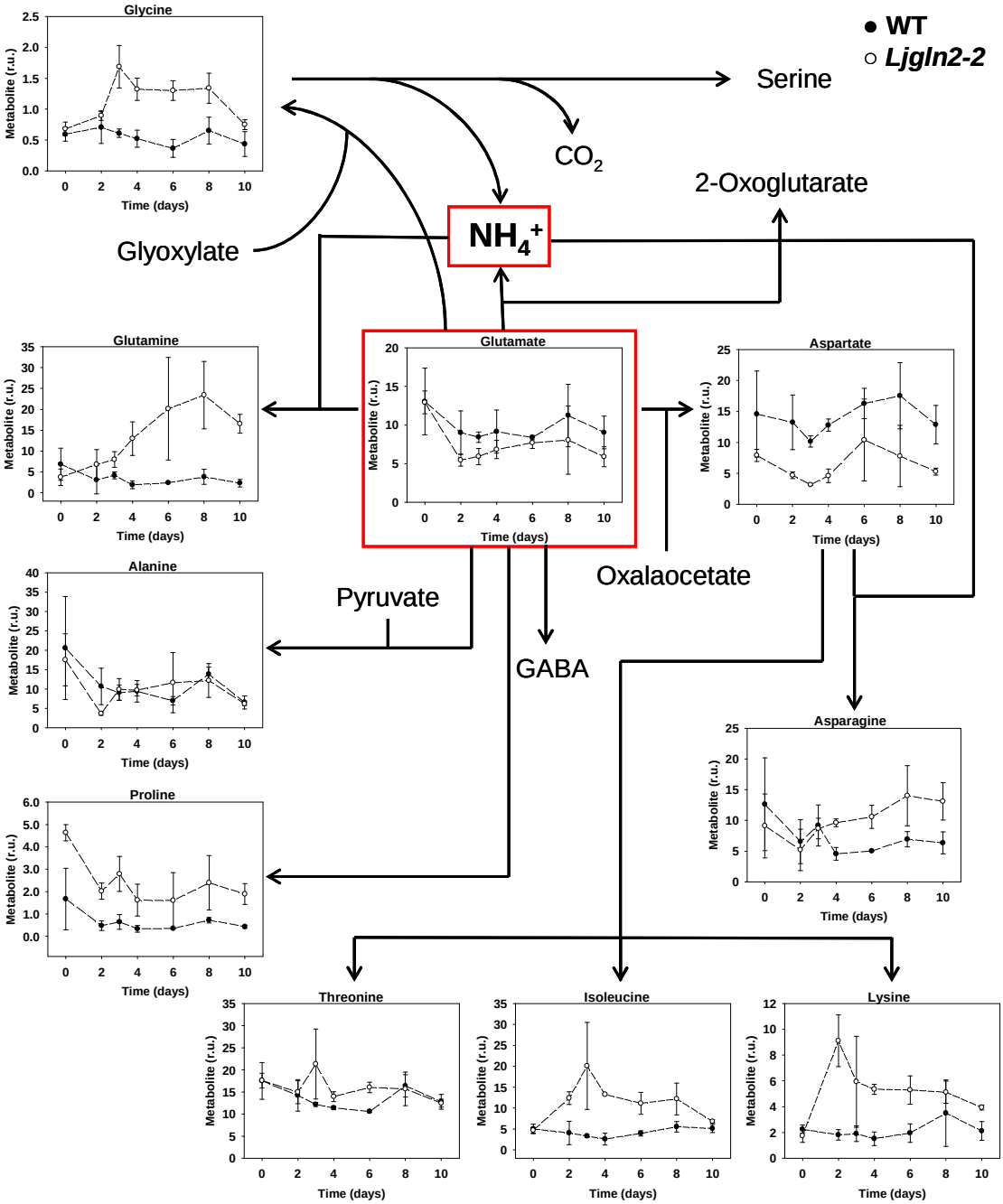


Fig. 6 Model for alternative NH_4^+ assimilation in *L. japonicus* under active photorespiratory conditions. For simplicity, the names of the enzymes involved that catalyze the reactions in the Figure are not reported. Methionine is also derived from Asp was not detected in the metabolomic experiment carried out by Pérez-Delgado et al. (2013).

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Publicación 3.

Transcriptomics of leaves of *Lotus japonicus* plants grown under different nitrogen regimes. Analysis of primary and secondary nitrogen assimilation.

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Unpublished.

ABSTRACT

Nitrogen (N) is one of the most important nutrients for plants and, in natural soils, its availability is often a major limiting factor for plant growth. Legume plants are able to grow using atmospheric N_2 fixed in root nodules or by using mineral sources of nitrogen. Primary nitrogen assimilation by plants involves the use of different forms of inorganic nitrogen (NO_3^- and/or NH_4^+). In addition, efficient secondary NH_4^+ assimilation must also exist in plants in order to reassimilate the ammonium ions that can be produced endogenously in the plants from processes such as photorespiration, being this ammonium as much as 10 times higher than the one trough primary assimilation. First, in this paper it was evaluated the effect of the different nitrogen sources on gene expression in leaves of the model legume *Lotus japonicus* and it was demonstrated that several important transcriptomic changes occurred when plants were cultivated with different nitrogen sources compared to purely symbiotic conditions, including genes involved in nitrogen, carbon and secondary metabolism and some transcription factors with a possible key role in the regulation of these processes. In addition, it was also studied the effect of the photorespiration and the deficiency of plastidic GS on gene expression in leaves of *L. japonicus.* This paper provides novel information of the possible role of plastidic GS in the response to different nitrogen sources and C/N balance of *L. japonicus* plants.The use of gene co-expression networks indentify possible transcription factors that may be involved in the response to different nitrogen nutritions and / or photorespiratory metabolism and the lack of plastidic GS.

INTRODUCTION

Nitrogen (N) is one of the most important nutrients for plants and, in natural soils, its availability is often a major limiting factor for plant growth. The use of nitrogen by plants involves several steps, including uptake, assimilation, translocation and different forms of recycling and remobilization processes, all of them of crucial importance in terms of nitrogen utilization efficiency (Hirel et *al.*, 2007; Masclaux-Daubresse et *al.*, 2010). Primary nitrogen assimilation by plants involve the use of different forms of inorganic nitrogen (NO_3^- and/or NH_4^+), depending on nitrogen availability, plant species and adaptations. Alternatively, the symbiosis with bacteria enables also to several plant species, most notably legumes, to use atmospheric N_2 which is reduced to NH_4^+ in the nodules by the action of bacterial nitrogenase. In addition, efficient secondary NH_4^+ assimilation must also exist in plants in order to reassimilate the ammonium ions that can be produced endogenously in the plants from processes such as photorespiration, phenylpropanoid biosynthesis, or amino acid catabolism (Betti et *al.*, 2012a).

Lotus japonicus is a temperate legume that can grow using the atmospheric N_2 fixed in the nodules or by using external sources of nitrogen, like nitrate or ammonium, in roots. The utilization of NO_3^- requires its reduction to NH_4^+ produced by the consecutive action of nitrate reductase (NR; EC 1.7.99.4) and nitrite reductase (NiR; EC 1.7.1.4) enzymes, prior to ammonium assimilation. Then, the NH_4^+ synthesized as a result of both primary and secondary assimilation is assimilated into glutamine and then into glutamate by the enzymes glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1 or EC 1.4.1.14). In addition to GS and GOGAT, which catalyse irreversible reactions, a third enzyme, glutamate dehydrogenase (GDH; EC 1.4.1.2/4) catalyses a reversible amination/deamination reaction, which could lead to either the synthesis or the catabolism of glutamate from 2-oxoglutarate and NH_4^+. In most temperate legumes, it is proposed that asparagine, rather than glutamine, is the principal molecule used to transport reduced nitrogen within the plant, in contrast to many other plant species (Credali *et al.*, 2013). This is the case for *L. japonicus* where it has been shown that asparagine can account for almost 90 % of the nitrogen transported from root to shoot (Waterhouse *et al,*. 1996). Asparagine synthetase (AS, EC 6.3.5.4) is the main enzyme in charge of asparagine biosynthesis in plants. This enzyme catalyzes the transfer of the amide group from glutamine (or, in minor proportion, ammonium) to aspartate in an ATP-dependent reaction. The presence of nitrogen is sensed by the roots and produces early local transcriptional changes. However, there are also long-range signals related with N availability that go on both ways, from root to shoot and viceversa.

Despite of the fact that more energy is needed for the assimilation of nitrate, most plants prefer NO_3^- over NH_4^+. With the exception of ammonium tolerant species, the availability of NH_4^+ alone as nitrogen source, as well as the internal production of NH_4^+ by processes like photorespiration (Keys *et al.*, 1978) may result toxic to the plant. Notably, the toxic effect of external NH_4^+ can be relieved by co-provision of nitrate, the so called mixed nutrition. A fascinating and still poorly understood aspect of nitrogen nutrition is that in most cases the growth of a plant on NH_4NO_3 can surpass the maximal growth compared to either NO_3^- or NH_4^+ alone. This relief of NH_4^+ toxicity by NO_3^- may be related to a synergism between the signalling routes of NH_4^+ and NO_3^- (Britto *et al.*, 2002). Moreover, several genes are modulated exclusively when the nitrogen source is NH_4^+, NO_3^- or NH_4NO_3 (Lopes and Araus, 2008; Ruffel *et al.*, 2008).

On the other hand, recent data suggested that not only primary nitrogen assimilation, but also the secondary ammonium produced by the photorespiratory cycle, can cause important modulation in the leaf transcriptome of *L. japonicus*, affecting several genes for nitrogen, secondary and central carbon metabolism (Pérez-Delgado *et al*., 2013). Photorespiration is the most important process in which high amounts of ammonium are released at a rate that can exceed by 10-fold the rate of primary nitrate assimilation in plants (Keys *et al*., 1978). Ammonium is produced as a result of the decarboxylation of two molecules of glycine to yield one molecule of serine in a reaction catalyzed by glycine decarboxylase (GDC) and serine-hydroxymethyl transferase (SHMT) enzymatic complex in the mitochondria. This ammonium is then reassimilated in the chloroplast by the plastidic glutamine synthetase (GS2) and ferredoxin-glutamate synthase (Fd-GOGAT). These reactions forms part of a more complex process called as the photorespiratory nitrogen cycle. Photorespiration starts by means of the oxygenase activity of RUBISCO in the chloroplasts, which produces 2-phosphoglycolate (a 2C compound), and is aimed to retrieve 3 out of each 4 carbon atoms entering this pathway as 2-phosphoglycolate in order to yield 3-phosphoglycerate which goes back to the Calvin cycle (Bauwe *et al.*, 2010; Keys *et al.*, 1978; Maurino and Peterhansel, 2010; Wingler *et al*., 2000). Several important pathways like nitrogen assimilation, respiration, one-carbon metabolism, purine biosynthesis (Bauwe *et al*., 2010) and redox signalling (Foyer *et al*., 2009) interact in different ways with photorespiration.

The first GS photorespiratory mutants isolated from legume plants were identified several years ago in our laboratory from the model legume *Lotus japonicus* (Orea *et al.*, 2002; Márquez *et al*., 2005). These mutants were shown to be specifically deficient in GS2 and have been substantially characterized at the molecular and physiological levels (Orea *et al.*, 2002; Márquez *et al.*, 2005;

Betti *et al*., 2006; Díaz *et al*., 2010; García-Calderón *et al.*, 2012; Betti *et al.*, 2012a; Pérez-Delgado *et al*., 2013). Under CO_2-enriched atmosphere, the mutants did not show any visible phenotype and only a slightly lower growth rate (Orea *et al.*, 2002).

In order to study the effect of the different nitrogen sources on gene expression in leaves of the model legume *L. japonicus*, a comparative transcriptomic study was carried out in leaves of plants grown with different nitrogen sources (NO_3^-, NH_4^+ or NH_4NO_3) or under conditions of biological nitrogen fixation. As the ammonium assimilated trough photorespiration may be 10 times higher than the one trough primary assimilation (Keys *et al*., 1978), it has, also, been evaluated the effect of the photorespiration and the deficiency of plastidic GS on gene expression in leaves of *L. japonicus*. This paper demonstrate that several important transcriptomic changes ocurred in leaves of *L. japonicus* when plants were cultivated with different nitrogen sources compared to purely symbiotic conditions, including genes involved in nitrogen, carbon and secondary metabolism and some transcription factors with a possible key role in the nitrogen signaling. Also, this paper provides novel information of the possible role of plastidic GS in the signaling of different nitrogen sources and in the signaling of the C/N balance of *L. japonicus*.

MATERIAL AND METHODS

Growth conditions and harvesting of plant material

Lotus japonicus (Regel) Larsen cv. Gifu (B-129-S9) was initially obtained from Professor Jens Stougaard (Aarhus University, Aarhus, Denmark) and then self-propagated at the University of Seville. The *Ljgln2-2* mutant, which lacks of plastidic GS2 protein and activity (Betti *et al.*, 2006) was isolated from the photorespiratory mutant screening carried out using ethyl methanesulfonate as described previously (Orea *et al.*, 2002). The mutant progeny of two consecutive backcrosses into the WT background were used. WT and mutant seeds were scarified and surface-sterilized, then germinated in 1% agar in Petri dishes and transferred to pots using vermiculite as solid support. Five seedlings were planted in each pot and grown until plants had 7 trifoils in a growth chamber under 16 h : 8 h day : night, 20 : 18°C, with a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1} and a constant humidity of 70%. When required, CO_2 was automatically injected to a final concentration of 0.7% (v/v) (high CO_2 photorespiration-suppressed atmosphere)

Nodulated plants had been inoculated with *M. loti* and were watered without a nitrogen source and supplemented with 3 mM KCl (Handberg and Stougaard, 1992). *M. loti* TONO JA76 (Kawaguchi *et al.*, 2002) was grown in YM liquid medium (Vincent, 1970) at 28°C to an optical density at 600 nm = 1, and was then collected by centrifugation for 30 min at 2,408 × g and was resuspended in 0.75% (wt/vol) NaCl. Once sown in the pots, the plants were inoculated by the addition of 2 ml of this bacterial solution.

Plants on different nitrogen nutritions were obtained either watering continuously with "Hornum" nutrient solution containing 10 mM KNO_3 (NO_3^- plants) or after the transfer for the last ten days from this solution for the to 10 mM NH_4Cl and supplemented with 3 mM KCl (NH_4^+ plants) or 5 mM NH_4NO_3 and 3 mM KNO_3 (NH_4NO_3 plants). After all plants reached the same size, in this case an average of 7 trifoils, leaf tissue was harvested for each plant genotype. Every harvest involved at least three independent biological replicates for each genotype and treatment. A biological replicate consisted of tissue pooled from five plants grown in the same pot.

Different sets of WT plants were grown continuously either in high CO_2 (0.7 % v/v) or normal air atmospheres. In the case of the *Ljgln2-2* mutant, the plants were grown continuously in high-CO_2 conditions.

RNA extraction and qRT-PCR

Leaf material was flash frozen in liquid nitrogen, homogenized with a mortar and pestle and kept at −80°C until use. Three independent biological replicates were used for the quantitative real-time RT-PCR (qRT-PCR) analysis. Total RNA was isolated using the hot borate method (Sánchez *et al.*, 2008). The integrity and concentration of the RNA preparations were checked using an Experion bioanalyzer (Bio-Rad) with RNA StdSens chips and a Nano-Drop ND-1000 (Nano-Drop Technologies), respectively.

For qRT-PCR analysis, total RNA was treated with the TURBO DNA-free DNAse (Ambion). Reverse transcription was carried out using SuperScript III reverse transcriptase (Invitrogen), OligodT and RNAsin RNAse inhibitor (Ambion). DNA contamination and RNA integrity were checked by carrying out qRT-PCR reactions with oligonucleotides that amplified an intron in the *LjHAR1* gene and the 3' and 5' ends of the *L. japonicus* glyceraldehyde-3-phosphate dehydrogenase respectively. qRT-PCR reactions were carried out in 10 μL in a Lightcycler 480 thermal cycler (Roche) using a SensiFAST SYBR No-ROX Kit

(Bioline). Expression data were normalized using the geometric mean of four housekeeping genes: *LjGPI*-anchored protein (chr3.CM0047.42), *LjPp2A* (chr2.CM0310.22), *LjUbc10* (chr1.TM0487.4) and *LjUbq* (chr5.CM0956.27) that were selected amongst the most stably expressed genes in plants (Czechowski *et al.*, 2005). A list of all the oligonucleotides used is provided in Supplemental Table S1.

DNA chip hybridisation and data analysis

Leaf material was flash frozen in liquid nitrogen, homogenized with a mortar and pestle and kept at −80°C until use. Two independent biological replicates were used for the transcriptomic analysis.

Microarray slides were designed and produced using Agilent eArray (Agilent Technologies; http://www.agilent.com) specifically developed for *L. japonicus*.

The microarrays were scanned, the raw image files were processed and data analysis was performed using Agilent Technologies. Data were normalized using the LIMMA library (Linear Models for Microarray Data, v. 2.10.5) (Smyth, 2004) of the bioconductor package. Differentially expressed genes were determined using Rank products (Breitling *et al*., 2004) passing a t test ($P < 0.1$) and the false discovery rate (FDR) correction.

The differentially expressed genes were visualised using the MapMan program (Usadel *et al*., 2005) and analysed according to the corresponding metabolic pathways or functional categories using Pathexpress (Goffard and Weiller, 2007). The default threshold of $P < 0.1$ for the FDR was used for Pathexpress. Gene sequence searches were carried out at the Kazusa database (http://www.kazusa.or.jp/lotus/) and at the TIGR gene index (http://plantta.jcvi.org/index.shtml).

Microarray data collection and preprocessing.

Microarray data used in this work was obtained from experiments published in: Sánchez *et al*., 2008, Høgslundet *et al*., 2009, Díaz *et al*., 2010, Sánchez *et al*., 2011, Betti *et al*.**,** 2012b and Pérez-Delgado *et al*., 2013. CEL files of these experiments are available in the public microrrays database EBI (https://www.ebi.ac.uk/arrayexpress/). Code numbers of experiments are: E-

MEXP-1204, E-TABM-715, E-MEXP-2344, E-MEXP-2690, E-MEXP-1726, E-MEXP-3710 and E-MEXP-3603. Background correction and norm, alization of the raw data sets was performed using Robust MultiChip Analysis (RMA) implemented in “affy” R package (Gautier *et al.*, 2004).

Identification of differentially expressed genes.

The non-parametric RankProduct method (Breitling *et al*., 2004) was used to identify differentially expressed genes between treatment and control conditions. A gene was considered statistically significant if its false discovery rate (FDR) adjusted p-value was equal or smaller than 0.05.

Network construction and co-expression module detection.

A weighted gene co-expression network was constructed using the WGCNA R package version 1.27.1 (Langfelder and Horvath, 2008) with differentially expressed genes. First, the Pearson correlation matrix was weighted by raising it to a power (β). To choose the appropriate power, the network topology for various soft-thresholding powers was evaluated using pickSoftThreshold function and β=6 was chosen because this ensured an approximate scale-free topology of the resulting network. Next, the pairwise measure of gene co-expression of the resulting weighted network was transformed into a topological overlap (TO) similarity measure (TO), which is a robust measure of pairwise interconnectedness (Yip and Horvath, 2007). A TO similarity measure between two genes (ij) is defined as: $TOij = \Sigma u\ aiuauj + aij$ $\min(ki,kj)+1-aij$ where $ki = \Sigma u\ aiu$ was the node connectivity and a is the network adjacency. Finally, TO similarity measure coupled with average linkage hierarchical clustering was performed for module detection using the Dynamic Tree Cut algorithm (Langfelder *et al*., 2008). The coexpression network was visualized using Cytoscape v 3.0 and analyzed using the NetworkAnalyser plugin (Doncheva *et al*., 2012). In order to simplify the display of the network and to focus on relevant relationships, only edges in this network of the corresponding TO similarity measure above a threshold of 0.11 are shown in Figures 1 and 4. Further, NetworkAnalyser plugin was used to assess which genes in the network form hubs.

RESULTS AND DISCUSSION

Analysis of genes differentially expressed in nodulated plants and in plants grown with external nitrogen sources.

A comparative transcriptomic study was carried out in leaves of plants grown with different mineral nitrogen sources (NO_3^-, NH_4^+ or NH_4NO_3) or under conditions of biological nitrogen fixation (Nod). A Rank Product analysis with FDR correction identified 609 differentially expressed probesets when comparing these conditions.

A gene co-expression network was constructed using the WGCNA R package with all the transcriptomic data available for *L. japonicus* to establish the degree of connectivity of each of the modulated genes and to try to determine its importance in the group analyzed. The co-expression networks of genes whose expression levels in plants supplied with external N were higher or lower than the expression levels in nodulated plants were visualized using Cytoscape and analyzed using the NetworkAnalyser (Doncheva *et al.*, 2012) (Fig. 1; Supplemental Table S2 and Supplemental Table S3).

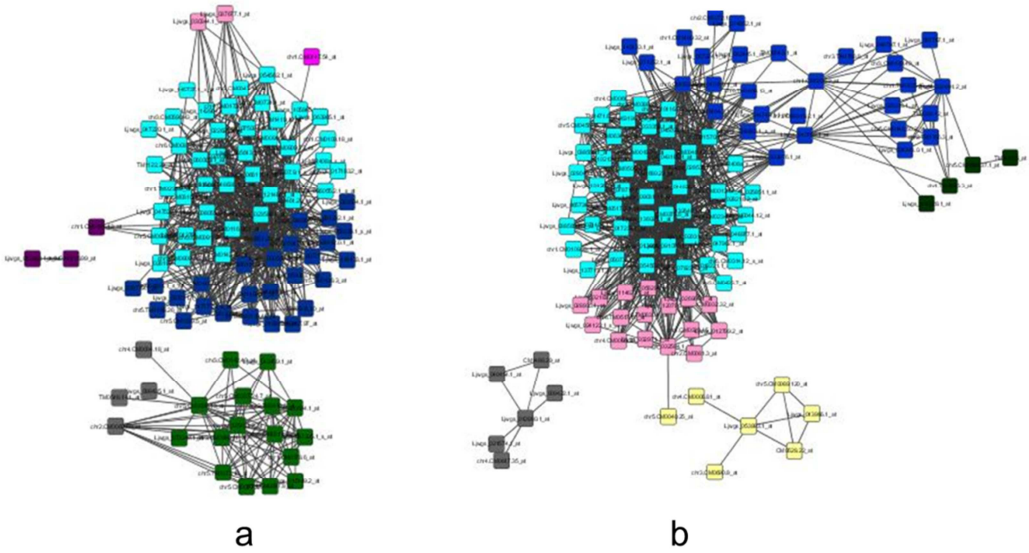

Figure 1. Networks analysis of genes that had higher (a) of lower (b) expression level in plants cultivated with any of the N mineral nutrition (NO_3^-, NH_4^+ or NH_4NO_3) compared with nodulated plants. The colored squares represent the modulated genes and the lines represent the connections among the above mentioned genes. Genes marked with the same color has a transcriptional regulation similar bearing all the experiments included to construct the network.

Genes with a major expression in plants cultivated with a mineral nitrogen source could be related to the metabolism and/or signalling produced by the external nitrogen and/or to the signalling or processes related with a suppressed

nodulation. In contrast, genes with a major expression in nodulated plants could be related to the promotion of nodulation and/or N_2 fixation.

Among the group of genes more expressed in plants cultivated with external nitrogen sources several genes stood out attending to its high degree of connectivity. First, there were several genes related to the nitrogen metabolism, such as one of the carriers of nitrate (probeset chr4.CM0161.9) and an asparagine synthetase (probeset TM1307.9.1). The modulation of this last gene is justified by the fact that asparagine can account for 86% of the nitrogen flux from root to shoot in *L. japonicus*, when the plant is adequately fed (Waterhouse *et al.*, 1996). Asparagine also plays an important role in the regulation of N flux in the N-organic pool (Lam *et al*., 1996) and may also act as a metabolite signal in the developing embryo and influence seed protein accumulation (Pandurangan *et al*., 2012; Credali *et al.*, 2013). In agreement with this, there were some modulated genes related with proteins implicated in seed maduration (probesets chr3.CM0996.11, chr5.CM0357.22.2, Ljwgs_027305.1 and Ljwgs_056638.1) and with late embryogenesis abundant protein (probesets Ljwgs_025401.2 and Ljwgs_015149.2). Moreover, there were some genes related to carbon metabolism that were more expressed in plants cultivated with mineral nitrogen sources, like a UTP-glucose glucosyltransferase (Ljwgs_015919.1) and a sucrose-phosphate synthase (chr3.CM0047.16), belonging both to starch and sucrose metabolisms respectively, thus confirming that a change in nitrogen nutrition affects carbon metabolism. Finally, there were some transcriptions factors with a high connection with some of the other genes which could have a relevant function in the signaling of nitrogen availability. The gene with the highest number of connection was a bHLH029 transcription factor (probeset Ljwgs_048638.1), whose ortholog gene in *Arabidopsis thaliana* is *At2g28160*. This gene is associated to Fe signaling (Meiser and Bauer, 2012). Also, there were others transcription factors as the OBF4 (also named TGA4 transcription factor; probeset chr5.CM0909.32) whose ortholog in *A. thaliana* is *At5g10030*. which has been recently associated to the nitrate response in roots of this plant (Álvarez *et al*., 2010). Curiously, there was, also, a modulation of two nodulins (probesets chr1.TM1305.1 and Ljwgs_142964.1) that belong to the family nodulin 21. There has been described recently that nodulines must play different roles in plant development; it has been identified a member of the plant-specific nodulin 21 gene family, designated WAT1 (*At1g75500*), which is essential for secondary wall formation in fibers of *Arabidopsis* (Ranocha *et al*., 2010).

Among the group of genes more expressed in nodulated plants several genes stood out attending to its high degree of connectivity. First, several genes related to redox metabolism were more expressed in nodulated plants. Among

this group there was a gene that encodes for a glutathione S-transferase (probeset Ljwgs_028218.2) and three genes of glutaredoxines (probesets Ljwgs_021839.1, chr1.CM0109.23.1 and chr3.CM0160.40). Also, there were four genes that encodes for Cytocrome P450, which belongs to xenobiotics metabolism. These data suggest the existence of a peculiar redox metabolism present in nodulated plants compared to plants cultivated with an external nitrogen source. Moreover, there were some genes related to carbon metabolism such as two sugar transporters (probeset Ljwgs_136027.1 and Ljwgs_065724.1) and four genes that encode for chinitase (probesets Ljwgs_091076.1, chr5.CM0456.15, Ljwgs_048977.1 and chr3.TM1768.8). It has been described that environmental changes may affect chinitase gene expression in *Arabidopsis thaliana* (Takenaca *et al.,* 2009). Likewise, there were changes in expression levels of genes involved in nitrogen metabolism, as a carrier of amino acids (probeset Ljwgs_028535.1), other one for ammonium (probeset Ljwgs_028040.1) and an asparaginase (probeset Ljwgs_021574.1). All these latter changes may be interesting because amino acid cycling was involved in nitrogen fixation in the legume-*Rhizobium* symbiosis (Lodwig *et al.,* 2003).. Finally, there were some transcriptions factors with high connections with other genes which could have a relevant function in the promotion of the nodulation. There was a WRK50 trasncription factor (probeset Ljwgs_012799.2), whose ortholog in *A. thaliana* is *At5g26170*, and two of WRK70 (probesets Ljwgs_120786.1 and chr1.CM0320.45), which have the same ortholog in *A. thaliana*, *At3g56400*. Both genes, *At5g26170* and *At3g56400*, are involved in defense responses. Also, there was a *NOD26* gene that is known for its role in nodulation.

To sum up, different groups of genes arose as a result of the previous analysis, including genes involved in nitrogen, sugar and secondary metabolism. Also, there were some transcription factors with high connections with other genes which could have a relevant function in the signaling of nitrogen availability. Many of the differentially expressed genes found have still an unknown function, which open new possibilities for future investigations.

Analysis of genes differentially expressed in plants grown with different mineral nitrogen sources.

A comparative transcriptomic study was carried out in leaves of plants cultivated with different mineral nitrogen sources using nodulated plants as a reference. A total of 832 differentially expressed probesets were identified in the three different nitrogen nutritions examined (Table 1). 223 of these probesets were the same among different treatments, and, therefore, a total of 609 probesets

were found to be differentially elicited when comparing the different nitrogen sources.

	Up	Down	Total
NO_3^-	38	153	191
NH_4^+	156	214	370
NH_4NO_3	145	126	271
Total	339	493	832

Table 1. Number of probesets differentially expressed in WT plants grown in different nitrogen sources compared with nodulated WT plants with FDR<0.1 with a Rank Product analysis.

Changes in gene expression were analysed by Rank Product applying a FDR < 0.1. No fold-change threshold was applied since recent transcriptomic studies in *L. japonicus* demonstrated that subtle transcriptional changes, if statistically significant, are often biologically relevant and represent robust responses to stress conditions. Statistically changed genes were visualized and functionally characterized using MapMan and PathExpress (Usadel *et al*., 2005; Goffard and Weiller, 2007). In most cases, the genes involved in these responses were specific to the local nitrogen source, as it happened in other studies (Ruffel et al., 2008). Each group of differentially expressed genes on each different nitrogen source is described below.

The first group, include genes that were more expressed in leaves of plants cultivated with nitrate than in leaves of nodulated plants. This group contain 38 probesets and the Pathexpress tool showed that “nitrogen metabolism” pathway was significantly over-represented in this group of genes, including the genes encoding for nitrite reductase (probesets chr4.CM0227.40 and gi9968472) and for one particular high-affinity NO_3^- transporter (chr4.CM0161.9) (Fig S1). *L. japonicus* assimilates NO_3^- mainly in the roots (Márquez *et al.*, 2005) but these data suggest that there are some NO_3^- -responsive genes in leaves.

Among less expressed genes in NO_3^--fed plants several pathways were over-represented such as those for phenylalanine metabolism and for stilbene, coumarine and lignin biosynthesis, belonging both to phenylpropanoid metabolism (Fig. S1). Genes encoding for key enzymes for phenylpropanoids and flavonoid biosynthesis were in this group, like phenylalanine ammonia lyase (probeset Ljwgs_078032.1), 4-coumarate-CoA ligase (probeset chr4.CM0061.26) and anthocyanin acyltransferase (probeset Ljwgs_018221.1). Flavonoids are a vast class of secondary metabolites involved in an ample number of processes, including plant–pathogen interactions, pollination, light screening, seed development and allellopathy. Moreover, most flavonoids show an important anti-oxidant capacity (Hernández *et al*., 2008). Many of the genes

involved in flavonoid biosynthesis are induced under biotic or abiotic stress. The higher expression of these genes in nodulated plants confirms previous results indicative of the existence of important differences in redox metabolism in nodulated plants compared to those cultivated with nitrate. Also, it is well known that flavonoids have a key role in nodulation (Falcone Ferreyra *et al*., 2012) and this may be also another explanation for the over-representation of this group of genes in nodulated plants.

Regarding to the up-regulatory changes in gene expression detected in leaves of plants cultivated with NH_4^+ it was found again that the nitrogen assimilatory pathway was over-represented compared to nodulated plants. On the other hand, there was an induction of glutathione metabolism (probests Ljwgs_030344.1 and Ljwgs_018510.1 encode for glutathione-S-tranferase) and flavonoid biosynthesis (Fig. S2), that could be related with a higfher level of oxidative stress present inplants growing under NH_4^+. These changes are possibly due to the toxicity generated under NH_4^+ nutrition in many plants (Britto *et al.*, 2002).

Among less expressed genes in NH_4^+ fed plants it has been found an over-representation of secondary metabolism. Key enzymes of flavonoid biosynthesis like PAL1 (probeset Ljwgs_078032.1) and 4-Coumarate:CoA ligase (probeset chr4.CM0061.26) were down-regulated (Fig. S2). These changes may also form part of the response of the plants to the presence of NH_4^+ as unique nitrogen source.

Under mixed nutrition, the main over-expressed pathways compared to nodulated plants were those corresponding to starch and sucrose metabolisms. This group included key genes for starch biosynthesis like starch synthase (probest chr5.TM0431.23) and starch branching enzyme (probeset chr1.CM0178.37), suggesting that the carbon assimilatory process was increased under these conditions (Fig. S3). It is also interesting to notice that probesets corresponding to two genes encoding for AS (probesets gi897770 and gi897772) in *L. japonicus* were up-regulated under mixed nutrition. Another interesting observation was that most of the nitrogen assimilatory genes were not over-represented under mixed nutrition in contrast to what it was observed for NO_3^- and NH_4^+-fed plants.

Among less expressed genes in plants cultivated in mixed nutrition, several genes related to secondary metabolism were found as well as genes for starch and sucrose metabolism, although, in this case, the genes were beta-glucosidases, which are more related to cell wall metabolism and ABA biosynthesis than with the main carbohydrates metabolism (Fig. S3).

As a summary of the transcriptomic results, it is concluded that important transcriptomic changes were observed in leaves of *L. japonicus* grown with different N sources compared to purely symbiotic conditions. Also, each nitrogen source had a specific response considering that only a minoritary proportion of genes were shared between more than one condition as also reported in other works (Lopes and Araus, 2008; Ruffel *et al*., 2008). It is very interesting to note that nitrate and ammonium nutritions reflected an over-representation of the genes involved in nitrogen metabolism, while the results obtained under mixed nutrition point out mainly genes related to carbon metabolism. In addition, it is also shown that there are important changes in genes for the secondary metabolism associated to the different nitrogen sources. In particular, NH_4^+-fed plants showed an over-representation of genes related to oxidative stress. The fact that secondary metabolites in legumes are very important for the establishment of symbiosis, especially flavonoids, suggests that the changes in secondary metabolites observed may be also related to the effects produced by the external nitrogen sources on nodulation, a topic that will require further investigation.

Influence of photorespiration on nitrogen nutrition: studies with *Ljgln2-2* mutants

Recent data from our lab showed that not only primary nitrogen assimilation but also the secondary ammonium produced by the photorespiratory cycle can cause an important modulation in the leaf transcriptome of *L. japonicus*, including changes in the expression of several genes for nitrogen metabolism (Pérez-Delgado et al., 2013). An experiment was performed to further investigate the influence of photorespiration on the different nitrogen nutritions examined before. For this purpose WT and *Ljgln2-2* plants were grown under these four nitrogen conditions previously studied but under either normal air atmosphere (active photorespiration) or high CO_2 (photorespiration suppressed conditions).

The expression levels of genes encoding for the different isoforms of glutamine synthetase, glutamate synthase, asparagine synthetase and glutamate dehydrogenase were determined by qRT-PCR. Fig. 2 shows the transcript levels found for some of the measured genes The transcript levels of genes of WT plants grown in high-CO_2 or air atmospheres were compared (Fig. 2a) together with those of WT and *Ljgln2-2* plants grown in a high-CO_2 atmosphere (Fig. 2b).

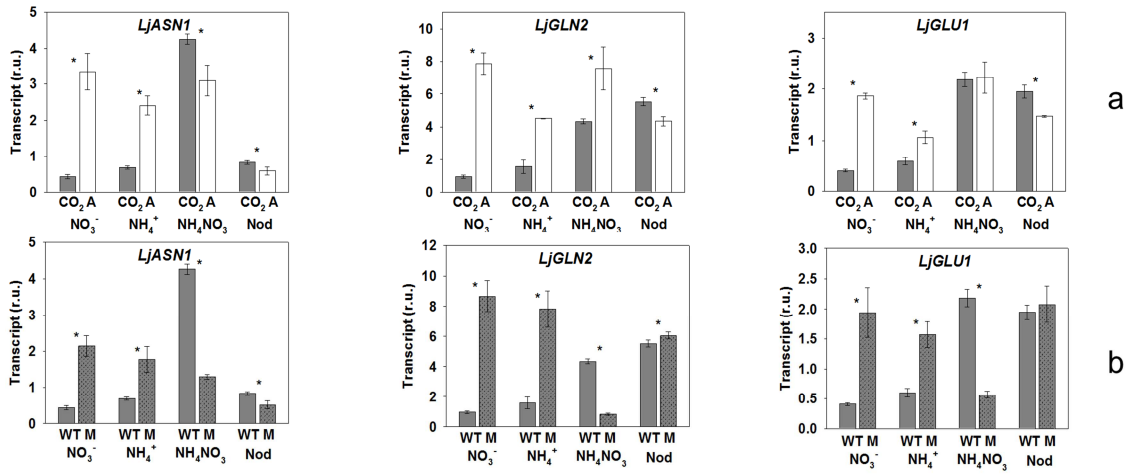

Figure 2. (a) Expression levels of some key genes of N metabolism in WT plants under normal air (A, white bars) or CO_2-enriched atmosphere (CO_2, grey bars) and different N sources. (b) Expression levels of the same genes in WT (WT, grey bars) and *Ljgln2-2* plants (M, grey dotted bars) grown under different nitrogen nutrition but in both cases in CO_2-enriched atmosphere. *LjASN1*: asparagine synthetase; *LjGLN2*: plastidic glutamine synthetase; *LjGLU1*: Fd-dependent GOGAT. Data are the mean ± SD of three independent biological replicates. *Indicates significant difference between high-CO_2 in a) and between WT and *Ljgln2-2* in b) determined by student's test ($P < 0.05$).

The results obtained indicated that, in all N-nutritions, there was a close similarity in the differential responses of WT plants in high-CO_2 versus air treatments (Fig. 2a) compared to WT versus *Ljgln2-2* mutant plants in high-CO_2 (Fig. 2b), suggesting that the deficiency in GS2 has a similar effect on the transcript levels than the diminishment of CO_2 concentration in the WT plants in all the nitrogen nutritions studied. Other studies also confirmed the clear association between plastidic GS and carbon metabolism in *L. japonicus* plants (Fig. 2) (Betti *et al.*, 2012a; García-Calderón *et al.*, 2012; Pérez-Delgado *et al.*, 2013).

In addition, it is interesting to notice that for the three genes analysed in Fig. 2B the transcriptional effects produced in the *Ljgln2-2* GS2-deficient mutant plants compared to the WT were very similar in the case of plants grown under NO_3^- and NH_4^+ but different, and in fact opposite to the effect produced in NH_4NO_3-grown plants. These results suggest a role for plastidic GS in the distinctive response to mixed nutrition of the nitrogen assimilatory genes in *L. japonicus*, and may imply a role of plastidic GS in the signalling events related to the presence of different nitrogen sources for the plant (see also Betti *et al.*, 2012a).

Study of the relation of the photorespiration and the deficiency of GS2.

The microarray data from the experiment published in Díaz *et al.*, 2010 and Betti *et al.*, 2012b which are available in the public microarrays database EBI with the accession codes E-MEXP-2690 and E-MEXP-3710 respectively were further explored to study more in detail the relationship between GS2 and carbon metabolism it was used. The transcriptomic changes produced in the WT by the diminishment of CO_2 atmospheric concentration were compared with the transcriptomic changes produced by plastidic GS deficiency in *Ljgln2-2* mutant plants grown under non-photorespiratory conditions (high CO_2).

1668 probesets were differentially modulated when comparing WT plants grown either in high CO_2 or normal air conditions, which means genes that can be modulated by carbon and/or active photorespiratory conditions. On the other hand, 1200 probesets were differentially expressed as a result of the lack of GS2 (FDR < 0.1). 288 probesets were modulated in both conditions (Fig. 3a). Interestingly, these commonly regulated probesets showed a strong linear correlation (r^2 = 0.91) between $\log_2$ of the fold change in gene expression between the two different comparisons carried out. In fact, the slope of the regression line was 0.9, indicating that the level of the changes in transcript levels were very similar in both conditions (Fig. 3b). Only 6% of genes, corresponding to 18 probesets, showed an opposite response to the diminishment of CO_2 concentration and the lack of GS2.

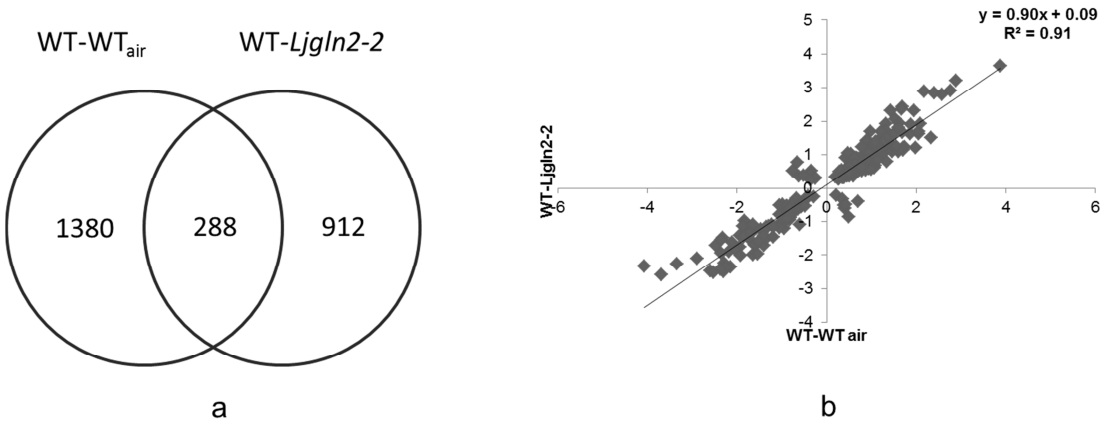

Figure 3. (a) Venn Diagram showing the number of probesets modulated by diminishment of CO_2 concentration and/or the absense of plastidic GS (FDR < 0.1). (b). Comparison of the $\log_2$ of fold change value for the probesets that are significantly elicited by both conditions.

A co-expression network was constructed using these 288 shared probesets previously analyzed (Fig. 4; Supplemental Table S4). Interestingly, many genes involved in carbon were visualized from this network. Also, other genes and transcription factors with important connections were detected.

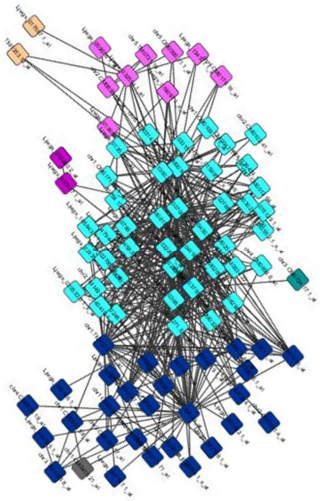

Figure 4. Networks analysis of genes modulated by the diminishment of CO_2 concentration and by the lack of GS2. The colored squares represent the modulated genes and the lines represent the connections of the above mentioned genes. Genes marked with the same color has a transcriptional regulation similar bearing all the experiments included to construct the network.

One particular gene stood out in the network attending to its high degree of connectivity. This was one gene for pyruvate decarboxylase (probeset Ljwgs_007060.2), one of the genes of the central carbon metabolism with a very high importance in all organisms and which has also been reported to have a role in stress tolerance (Pinhero *et al.*, 2011). Other genes related to carbon metabolism were found such as a sugar transporter (probeset Ljwgs_136027.1), a diacylglycerol kinase (probeset Ljwgs_067758.1) and an alpha-galactosidase (probeset Ljwgs_025958.1), amongst others. In addition, several other genes were noted as those for for glutathione S-transferase (probesets Ljwgs_037557.1 and Ljwgs_027688.1), cytochrome P450 (probesets chr1.CM0591.58 and Ljwgs_099719.2) and glutaredoxine (probeset chr1.CM0109.23.1), suggesting the interconnection of carbon, photorespiration and the lack of GS2 with redox metabolism and/or stress responses. It is very interesting to note that there was a modulation of a glycolate oxidase (*LjGO1*) gene (probeset Ljwgs_013523.1) with a high number of connections with the other modulated genes. This gene has recently been described to may have a role in stress response in *L. japonicus* in relation with photorespiratory ammonium accumulation (Pérez-Delgado *et al.*, 2013). Finally, there were several transcription factors modulated with high connectivity like a bHLH transcription factor (probeset Ljwgs_015129.1) and a zinc finger protein (probeset Ljwgs_105843.1.1). These transcription factors may be of crucial importance in the C/N balance and photorespiratory metabolism of *L. japonicus* plants in relation to the crucial role of GS2 found in both processes.

Previous studies showed that most of the genes differentially expressed in *Ljgln2-2* and WT plants were elicited by drought stress specifically in the *Ljgln2-*

2, thus confirming the existence of a relationship between GS2 and the stress responsive machinery in *L. japonicus* (Diaz *el at.,* 2010). The present study established a novel link between the plastidic GS and the response to changes in external CO_2 provided to the plants, which further illustrates the relationship among GS2 and carbon metabolism in *L. japonicus* plants. The results shown in this paper emphasize how a defect in nitrogen assimilation affects carbon metabolism in leaves as it was also concluded in other previous studies in regard to the nodulation of this plant.

CONCLUSION

In summary, in this paper, it was demonstrated that several important transcriptomic changes occurred in leaves of *L. japonicus* when plants were cultivated with different nitrogen sources compared to purely symbiotic conditions, including genes involved in nitrogen, carbon and secondary metabolism and some transcription factors with a possible key role in the nitrogen signalling like bHLH029, TGA4, two nodulins of the family nodulin21, WRKY50 and two WRKY70. This study, also conclude, that each nitrogen source had a specific response in leaves of *L. japonicus*. Also, the study of the *Ljgln2-2* mutants, under non photorespiratory conditions, gave several novel insights on the possible role of plastidic GS in the signaling events related to the presence of different nitrogen sources and in the signaling of the C/N balance of *Lotus japonicus.*

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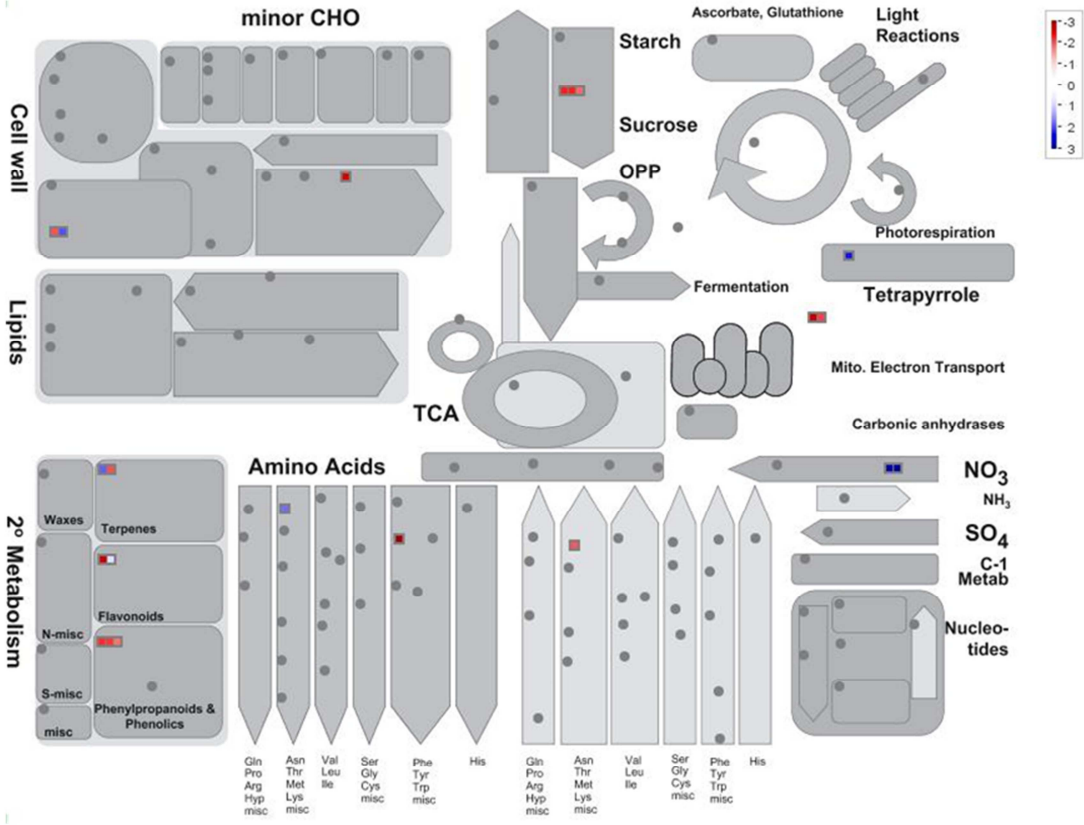
Supplemental Figure S1. MapMan metabolism overview and Pathexpress analysis of over-represented pathways of probesets elicited in leaves of plants grown with NO_3^- nutrition compared to nodulated plants. Changes in gene expression were analysed by Rank Product applying a false discovery rate (FDR) < 0.1. Red and blue indicate lower and higher expression than the control, respectively.



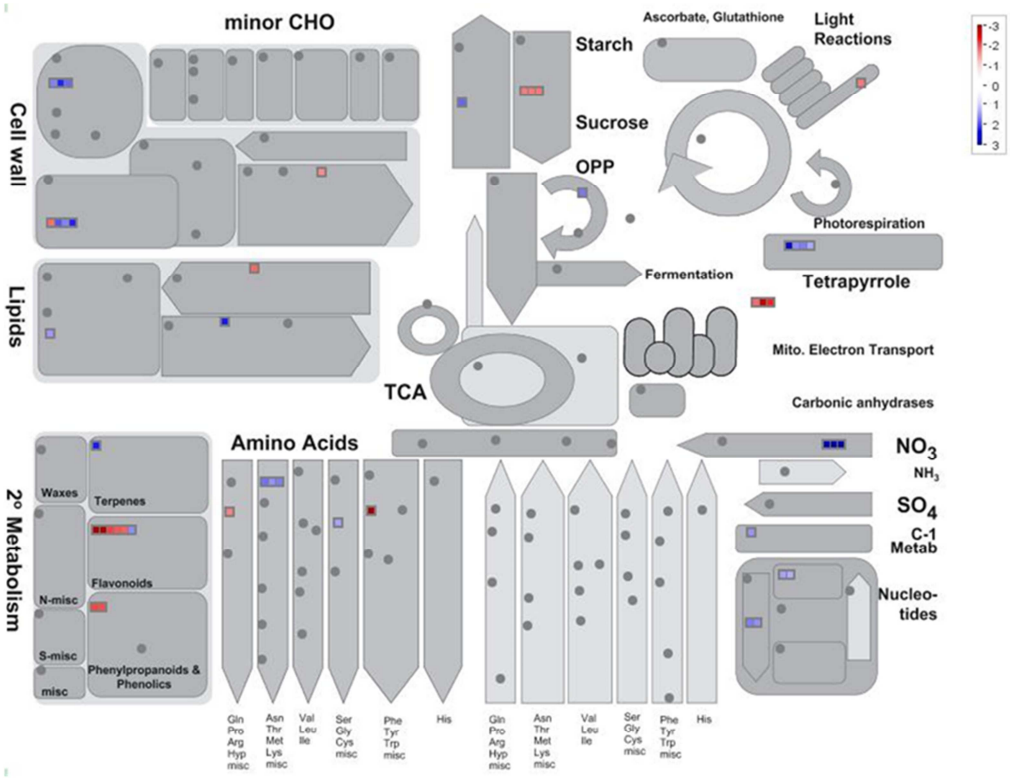
Up NO_3^-

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted
Nitrogen metabolism	map	23	2
1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)	map	3	1
Fluorene degradation	map	3	1

Down NO_3^-

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted
Stilbene, coumarine and lignin biosynthesis	map	10	3
Phenylalanine metabolism	map	14	3
gamma-Hexachlorocyclohexane degradation	map	9	2
Biosynthesis of 12-, 14- and 16-membered macrolides	map	1	1

Supplemental Figure S2. MapMan metabolism overview and Pathexpress analysis of over-represented pathways of probesets elicited in leaves of plants grown with NH_4^+ nutrition compared to nodulated plants. Changes in gene expression were analysed by Rank Product applying a false discovery rate (FDR) < 0.1. Red and blue indicate lower and higher expression than the control, respectively.



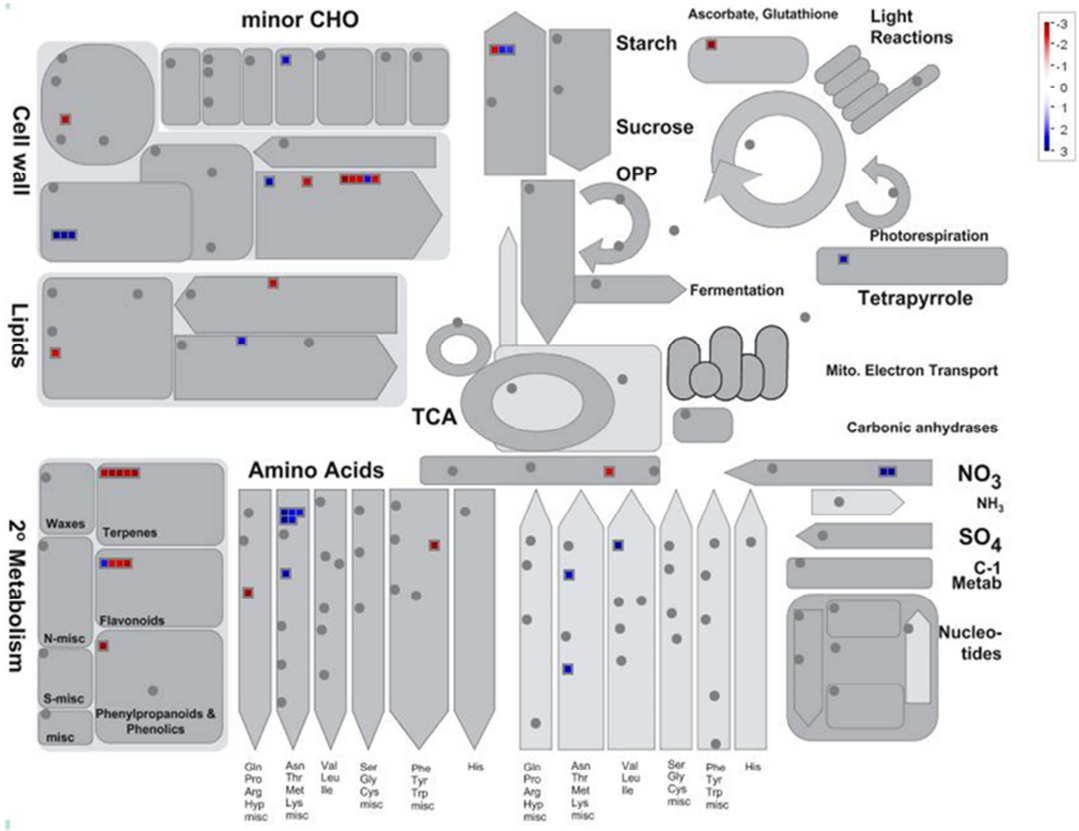
Up NH_4^+

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted
Nitrogen metabolism	map	23	3
Glutathione metabolism	map	11	2
Flavonoid biosynthesis	map	15	2
Glycosphingolipid biosynthesis - neo-lactoseries	map	3	1
Glycosylphosphatidylinositol(GPI)-anchor	map	3	1

Down NH_4^+

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted
Indole and ipecac alkaloid biosynthesis	map	5	3
Flavonoid biosynthesis	map	15	4
Alkaloid biosynthesis II	map	8	2
Biosynthesis of 12-, 14- and 16-membered macrolides	map	1	1
gamma-Hexachlorocyclohexane degradation	map	9	2
Stilbene, coumarine and lignin biosynthesis	map	10	2

Supplemental Figure S3. MapMan metabolism overview and Pathexpress analysis of over-represented pathways of probesets elicited in leaves of plants grown with NH_4NO_3 nutrition compared to nodulated plants. Changes in gene expression were analysed by Rank Product applying a false discovery rate (FDR) < 0.1. Red and blue indicate lower and higher expression than the control, respectively.



Up NH_4NO_3

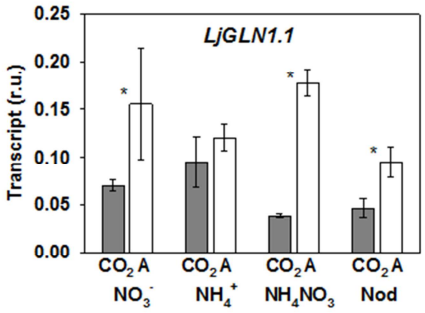
Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted
Starch and sucrose metabolism	map	31	4
Biosynthesis of 12-, 14- and 16-membered macrolides	map	1	1
Diterpenoid biosynthesis	map	12	2
1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)	map	3	1

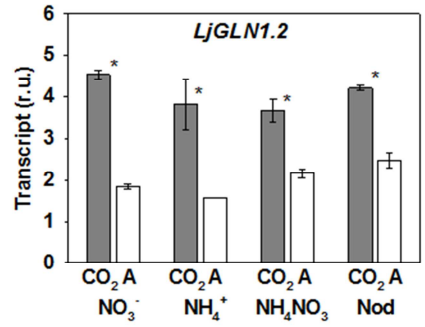
Down NH_4NO_3

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted
Indole and ipecac alkaloid biosynthesis	map	5	2
Starch and sucrose metabolism	map	31	4
Ascorbate and aldarate metabolism	map	8	2
Cyanoamino acid metabolism	map	9	2
Lipopolysaccharide biosynthesis	map	10	2

Supplemental Figure S4. (a) Expression levels of some key genes of N metabolism in WT plants under normal air (A, white bars) or CO_2-enriched atmosphere (CO_2 ,grey bars) and different N sources. (b) Expression levels of the same genes in WT (WT, grey bars) and *Ljgln2-2* plants (M, grey dotted bars) grown under different nitrogen nutrition and in CO_2-enriched atmosphere. *LjGLN*: cytosolic glutamine synthetase; *LjGLT*: NADH-glutamate synthase; *LjGDH*: glutamate dehydrogenase; *LjASN*: asparagine synthetase. *Indicates significant difference between high-CO_2 and air in a) and between WT and *Ljgln2-2* in b) as determined by student's test ($P < 0.05$).

(a)


LjGLN1.1
Transcript (r.u.)
0.25
0.20
0.15
0.10
0.05
0.00
CO2 A NO3-
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod


LjGLN1.2
Transcript (r.u.)
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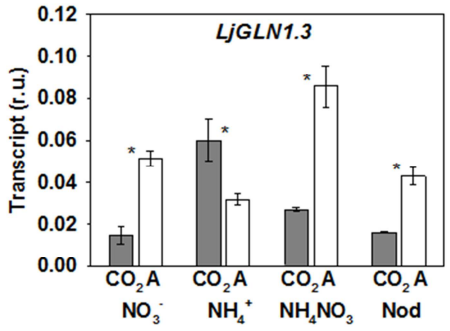
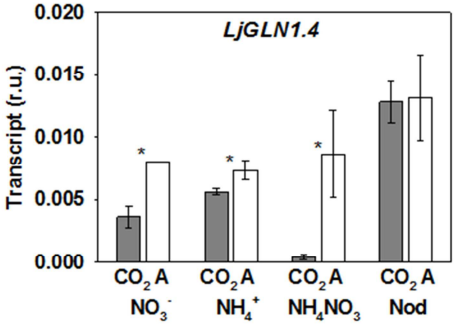
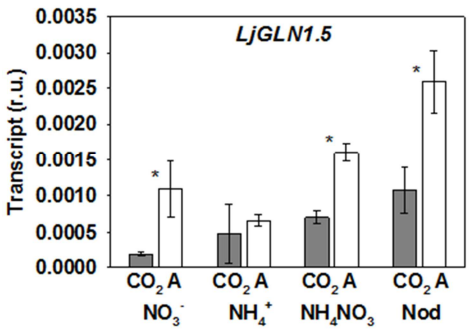
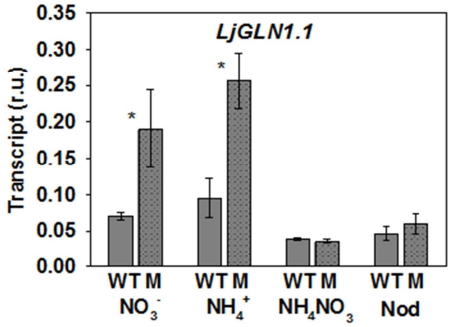
5
4
3
2
1
0
CO2 A NO3-
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod


LjGLN1.3
Transcript (r.u.)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
CO2 A NO3-
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod


LjGLN1.4
Transcript (r.u.)
0.020
0.015
0.010
0.005
0.000
CO2 A NO3-
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod


LjGLN1.5
Transcript (r.u.)
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
CO2 A NO3-
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod

(b)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0.25
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0.15
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0.05
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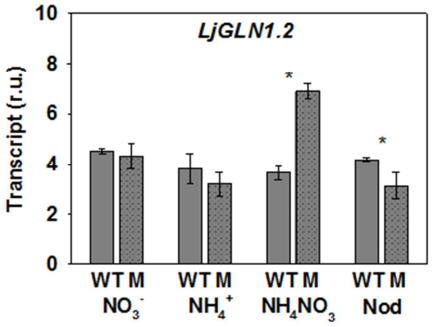
WT M NO3-
WT M NH4+
WT M NH4NO3
WT M Nod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Transcript (r.u.)
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4
2
0
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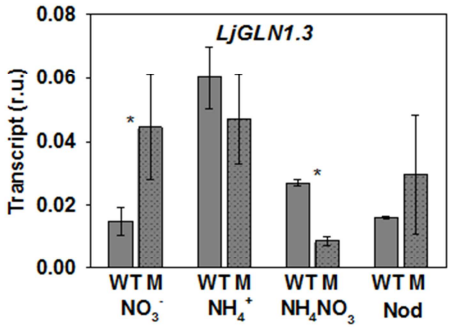
WT M NH4+
WT M NH4NO3
WT M Nod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0.04
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0.00
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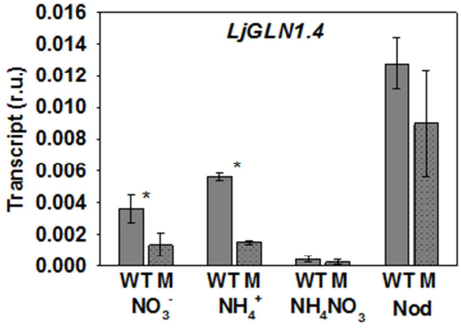
WT M NH4+
WT M NH4NO3
WT M Nod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0.012
0.010
0.008
0.006
0.004
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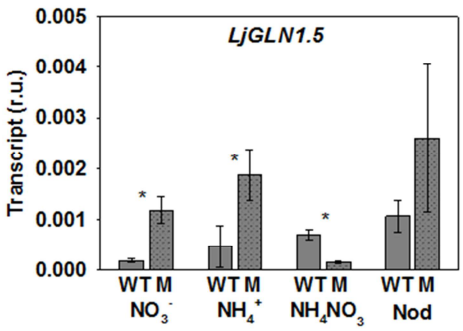
WT M NH4+
WT M NH4NO3
WT M Nod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Transcript (r.u.)
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0.003
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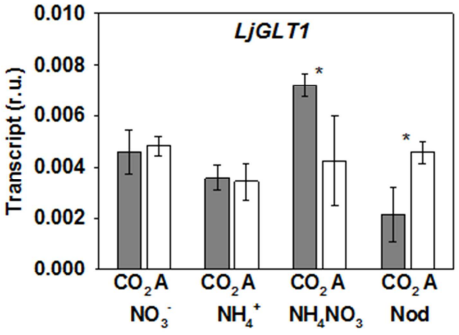
WT M NH4NO3
WT M Nod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Transcript (r.u.)
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0.006
0.004
0.002
0.000
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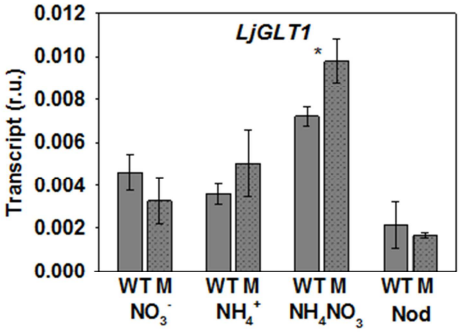
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod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Transcript (r.u.)
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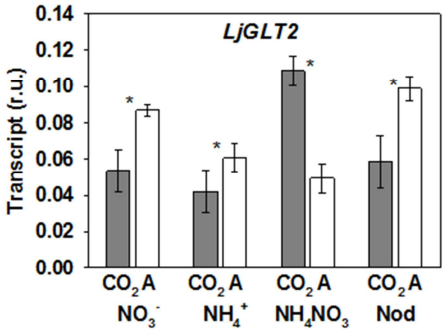
0.008
0.006
0.004
0.002
0.000
WT M NO3-
WT M NH4+
WT M NH4NO3
WT M Nod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Transcript (r.u.)
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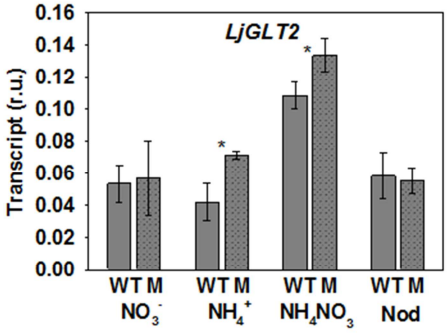
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod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Transcript (r.u.)
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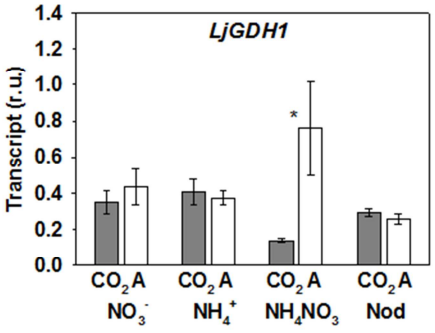
WT M NH4+
WT M NH4NO3
WT M Nod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Transcript (r.u.)
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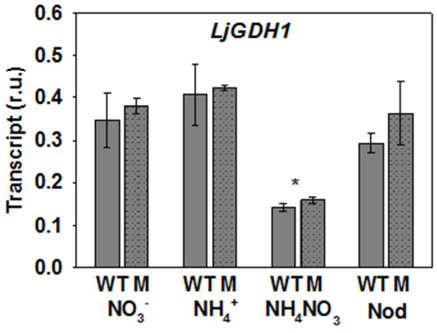
CO2 A NO3-
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod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Transcript (r.u.)
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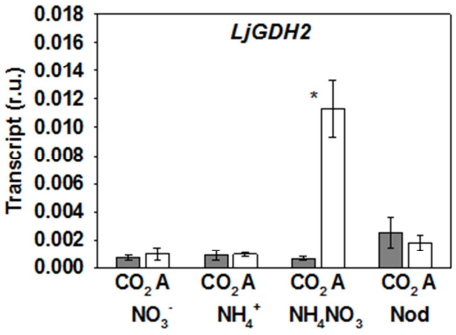
WT M NH4+
WT M NH4NO3
WT M Nod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Transcript (r.u.)
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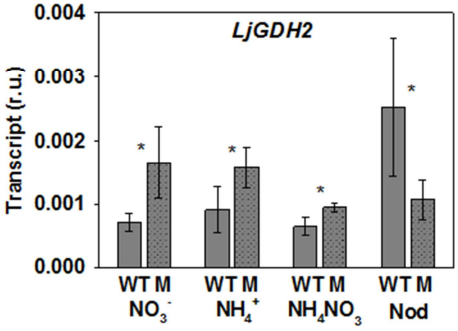
0.000
CO2 A NO3-
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod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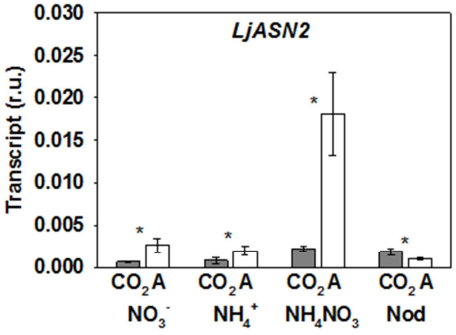
WT M NH4NO3
WT M Nod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Transcript (r.u.)
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0.000
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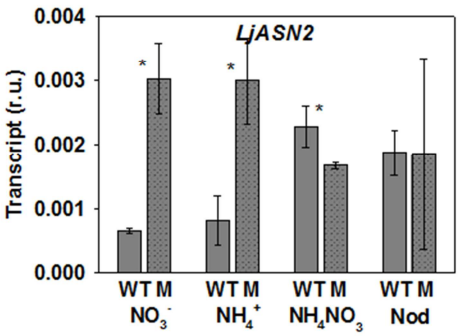
CO2 A NH4+
CO2 A NH4NO3
CO2 A Nod


LjASN2
Transcript (r.u.)
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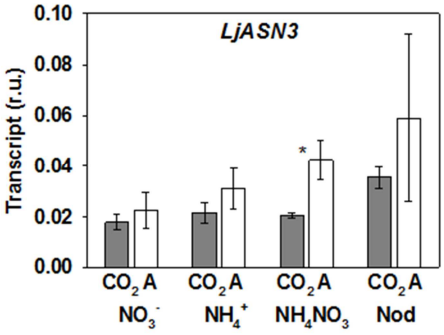
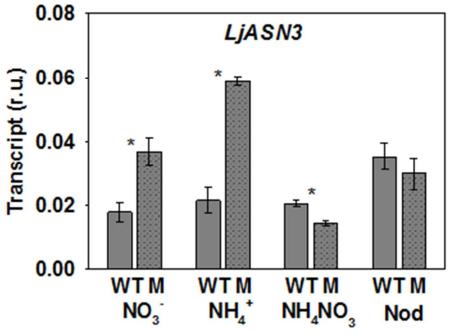
0.003
0.002
0.001
0.000
WT M NO3-
WT M NH4+
WT M NH4NO3
WT M Nod





Supplemental Table S1. Oligos for qRT-PCR measurements.

Gene	Kazusa 2.5 code	Primers	
		Forward	**Reverse**
LjGLN1.1	*chr2.CM0312.1480.r2.m*	TGGACCACAGGGCCCATAC	AATGTCACGCCCATAGGCTTT
LjGLN1.2	*chr6.CM0014.300.r2.m*	TGAGGTGTGGGTTGCTCGTT	AAGGACCACCCCAGCAATCT
LjGLN1.3	*LjSGA_030247.1*	TAACCTCTCCGAGACCACCG	CCAATCCATATGTATTCGGCG
LjGLN1.4	*LjSGA_058827.1*	AGAGAGACTGAGAAAGATGGAAAATGT	CAGGCCTCCTCTGTCCTCAA
LjGLN1.5	*LjSGA_019428.1*	GGGTAGGCAGGGAGACTGAAA	GAAGCTGGCCTCCTGTCCTC
LjGLN2	*chr6.CM0139.890.r2.m*	GGAAGAGGGAGGCTTTGAGGT	CTGGTGGCGAAGGGATAGATT
LjGLU1	*chr1.CM0009.170.r2.d*	ATTCAACGAGTAACAGCGCCA	CATGGGCTTCAATAAGGCTTCTC
LjGLT1	*LjSGA_035611.1*	TGGTGCTGATGCTATATGCCC	GTCAACCTGCAGTCGCCAA
LjGLT2	*LjSGA_037992.1*	TTGGCAATGTGGCACTGTATG	GCTGCCATCCCGTTGAAAT
LjGDH1	*chr1.CM0104.2530.r2.m*	GGAGATGTGCAAAACCCATGA	CGGTTAACTCCCAGGGTGAAA
LjGDH2	*chr4.CM2142.210.r2.a*	ACATGACAATGCTCGTGGTCC	AGGGTCAACCTCAGGGTGGTA
LjASN1	*chr5.CM0071.330.r2.d*	TGGAGGACCAACTGTTGCATG	AGACCAAGCAGCATCCCACTC
LjASN2	*LjT47C13.80.r2.d*	TCAGTGAGCAAAGGTGTTGAACC	CAAGAGGAGAAACTTCCATCTTGG
LjASN3	*LjT09J04.190.r2.d*	CGAACTGGCAGTGATTGTGAAGTG	ATGCCAATAGCATCACGAGCAG

Supplemental Table S2. List of genes that had higher expression level in plants cultivated with any of the N mineral nutrition (NO_3^-, NH_4^+ or NH_4NO_3) compared with nodulated plants. It is available in the electronical version.

Supplemental Table S3. List of genes that had lower expression level in plants cultivated with any of the N mineral nutrition (NO_3^-, NH_4^+ or NH_4NO_3) compared with nodulated plants. It is available in the electronical version.

Supplemental Table S4. List of genes elicited by the diminishment of CO_2 concentration and by the lack of GS2. It is available in the electronical version.

Publicación 4.

Abiotic stress in Lotus: aluminum and drought.

Pal'ove-Balang, Betti M, Díaz P, Pérez-Delgado CM, García-Calderón M, Monza J, Márquez AJ (2014). En: *Molecular approaches in Plant Abiotic Stress* (Gaur RK, Sharma P, eds). CRC Press, Boca Raton, Florida, USA, pp. 291-303.

Cf. Apartado titulado "Drought stress"

ABSTRACT

Different species of *Lotus* are currently used to improve pastures and hay quality where other forage legume species are not suitable, particularly in a diverse range of landscapes, including some often subjected to extreme environments and soil conditions. Among the forage legumes, *Lotus* sp. is moderately tolerant in areas constrained with low pH and Al toxicity. Cellular responses to Al toxicity are analyzed in *Lotus*, with an special focus to the changes produced at the root plant cell level, oxidative stress and other metabolic processes, as well as the possible role of proline, tannins and/or phytochelatins. On the other hand, drought stress is also another interesting topic of research in *Lotus* because it has been shown to substantially affect the productivity and persistence of *Lotus* species used in forage production. The recent transcriptomic and metabolic data obtained regarding drought stress responses in *Lotus* are summarized. Work carried out with either model or cultivated *Lotus* species and mutants is described, emphasizing the potential of results obtained with the model legume *L. japonicus.*

1. INTRODUCTION

Numerous abiotic impediments continue to limit yield potential in legumes, including: drought, soil salinity, acidity, aluminum and nutrient limitation. Legumes account for approximately a third of the world's primary crop production, human dietary protein and processed vegetable oil. Considering that legumes are second after grasses in importance to agriculture and cover around 15 % of farmed land, there is a crucial need to increase stress tolerance in legumes whether by traditional breeding or molecular engineering (Udvardi et al., 2005; Graham and Vance, 2003).

Among legumes, the genus *Lotus* includes more than 100 species that are found world wide except in very cold regions and the low land tropical areas of Southeast Asia and Central America. This worldwide distribution is partially due to their introduction to non-native areas by human activities and its adaptability to different environmental stresses. The adaptive characteristics shown by several *Lotus* species make them good candidates for restoration and phytoremediation of degraded environments (Belesky, 1999; Blumenthal and McGraw, 1999). Different species of Lotus are currently used to improve pastures and hay quality where other forage legume species are not suitable, particularly in a diverse range of landscapes, including some often subjected to extreme environments and soil conditions (Díaz et al., 2005a; Escaray et al., 2012). Consequently, the knowledge of the specific responses of *Lotus* plants to various types of stress and the corresponding molecular mechanisms lying down these responses, as well as the possible improvement of stress performance, is a subject of extraordinary interest. Recent projects, such as the European Union-funded project LOTASSA, coordinated by Drs. Juan Sanjuán and Monica Rebuffo, were aimed to join facilities, expertise and efforts in this direction (Rebuffo et al., 2008; LOTASSA, 2010).

The most important *Lotus* species from an agronomical point of view are *Lotus corniculatus* L. (birds-foot trefoil), *Lotus uliginosus* Schkuhr. (greater lotus), *Lotus tenuis* Waldst et Kit. (narrowleaf trefoil, also called *Lotus glaber* Mill.) and *Lotus subbiflorus* Lagasca (hairy birdsfoot trefoil, also denoted for many years as *Lotus pedunculatus*). Moreover, *L. corniculatus* is considered one of the major forage legumes after lucerne (*Medicago sativa*) and white clover (*Trifolium repens*). Main regions where *Lotus* species are exploited for agronomical purposes are South America, North America and Europe. 10 countries sow about 95 % of *Lotus* species in the world, and more than 90 % of this area is planted with *L. corniculatus*. However, *L. tenuis* is being increasingly used for forage production in temperate or subtemperate areas in Argentina (particularly in the Salado region), Chile, Uruguay and USA, mostly in Western

and Northeastern states. In turn, *L. uliginosus* is sown in New Zealand and coastal Sotheast Australia, whereas *L. subbiflorus,* the only annual *Lotus* species with agronomical importance, is primarily sown in Uruguay (Díaz et al.,2005a ; Escaray et al., 2010).

Lotus breeding programs can be assisted by the utilization of other closely related *Lotus* model legume species such as *L. japonicus* (ecotypes Gifu and MG-20), *L. filicaulis,* and *L. burtii*, as a result of recent advancements (Udvardi et al., 2005; Márquez, 2005) and has produced recently major breakthroughs in legume plant molecular biology (Udvardi et al., 2005; Stacey et al., 2006). Studies on most of the major leguminous crops are hampered by large genome sizes and other disadvantages (polyploidy, transformation or regeneration recalcitrancies, few or large seeds and seedlings, genome duplications, long generation times, etc). The work with model legumes overcomes many of these problems. Therefore, work with abiotic stress in *Lotus*, and particularly in the model legume *L. japonicus*, may be also extremely helpful not only for *Lotus* but also for the general progress achievements that can be obtained in other legume plants.

In this chapter we summarize the results recently obtained concerning two of the major sources of abiotic stress that affect cultivated *Lotus* plants worldwide: that is, aluminum and drought.

2. ALUMINUM STRESS

2.1. Al toxicity

Aluminum is the third most abundant element in the Earth crust and exists in soils in various forms that are in correlation with pH. In mineral soils at neutral pH it is present as insoluble aluminosilicates, aluminophosphates and hydroxyoxides. In acidic conditions (pH < 5.0) dissolution of Al is enhanced into the soil solution as $Al(H_2O)_6^{3+}$ that is strongly toxic for plants (Kinraide et al.,1991). The root is the most easily affected part of the plant by Al and inhibition of root elongation is one of the most evident badges of Al toxicity. Al strongly interacts with cell membranes and is also toxic for a wide range of cellular and metabolical processes including synthesis of nuclear DNA (Inostroza-Blancheteau et al., 2012). Cultivated pastures often occur in areas constrained with low pH and Al toxicity. Among the forage legumes, *Lotus* sp. is moderately tolerant to low pH in the soils (Blumenthal and Mc Graw 1999), therefore it is not only an interesting model plant for Al-stress response studies,

but also a promising species for improvements of the pastures productivity where other forage legumes are not suitable (Díaz et al.,2005a).

2.2. Cellular responses of Lotus to Al toxicity

The most extensive root injury caused by Al occurs in the distal transition zone in the root tip, where the cell division terminated and the cells are under preparation of a rapid elongation, but root cap, meristem and elongation zone are also sensitive and can accumulate Al (Sivaguru 1998, Panda et al., 2009). The highly reactive Al compounds are able to bind to several sites including cell wall, plasma membrane, cytoskeleton and also nucleus. The most affected are rhizodemal and cortical cells. The primary binding sites of Al^{3+} in the apoplast are probably the negative charged groups in the cell wall, such as pectins, xyloglucans or proteins that may immobilise about up to 99.9 % of the total Al fraction in root tissue (Poschenrieder 2008). However, the rate of Al accumulation is dependent not only on the pectin content, but also on its negative charge determined by its degree of methylation (Horst et al 2010).

In the cell wall, insoluble $Al_4(PO_4)_3$ complexes can be formed to retard the transport of Al but also inorganic P into the cytosol, causing phosphate deficiency symptoms (Zheng and Yang 2005) and activation of some isoenzymes of root acid phosphatase in model *Lotus japonicus* (Zelinová et al.,2009). Increase of activity and expression of acid phosphatase as well as its secretion are responses to phosphate deficiency which help in the mobilisation of Pi from organophosphates (Wasaki et al., 2009).

Inhibition of root elongation is a primary and rapid symptom of exposure of plant roots to Al. Although different mechanisms can be involved including strong binding of Al to pectins (Horst et al.,2010), inhibition of auxin transport into root apices (Kollmeier et al.,2000) or disruption of cytosolic Ca^{2+} homeostasis (Zhang and Rengel 1999), recent findings suggest an important role of ethylene evolution. In *Lotus japonicus*, similarly strong inhibition of root elongation was observed by Sun et al., (2007) after exposure to Al, ethylene-releasing substance, ethephon or to ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). The Al^{3+} induced inhibition of root elongation was substantially ameliorated in the presence of antagonists of ethylene biosynthesis, Co^{2+} and aminoethoxyvinylglycine. Furthermore, in the same work, the overexpresion of two genes for the key enzymes of ethylene synthesis, *MtACS* and *MtACO* was proved in *Medicago truncatula*, because the homologues gene

sequence data for *Lotus japonicus* were not available. Proteomic profiling in *Lotus* suggested also a disruption of cytoskeleton due to decline of α and β tubulin (Navascués et al., 2012), that have important function in cell wall synthesis and organisation (Wasteneis et al., 2004).

The common structural response to Al observed in *Lotus* sp. is an increase of vacuolar volume in young meristematic cells, formation of strong cell wall protuberances and callose depositions in the cell wall (Pal'ove-Balang et al., 2012). Callose deposits may accumulate in plasmodesmata, thus blocking the cell to cell trafficking, as it was postulated by Sivaguru et al., (2000). In *Lotus*, cell wall protuberances and callose deposits are not uniformly present throughout the cortex, but are irregularly distributed, with decreasing frequency centripetaly. Formation of such structures is likely related to cell defence mechanism to Al and has never been observed in low pH stress alone. The cells without such structures were deadly damaged (Pal'ove-Balang et al., 2012). One possible explanation of this phenomenon is a programmed cell death that was previously observed in barley (Pan et al., 2001). Mosaic-like structure of cell damage can affect the results of several physiological measurements, if performed in root-tip extracts.

Inorganic Al has a very strong affinity for the plasma membrane, where interacts with carboxylic groups, phosphate groups and lipids. Membrane bound Al causes rigidification of membranes and can stimulate Fe^{2+}-supported lipid peroxidation through binding to the membrane and promotion of changes in the arrangement of membrane lipids including packing of fatty acids that will facilitate the propagation of lipid peroxidation (Oteiza 1994). Plasma membrane potential has a crucial role for ion homeostasis of the cells and nutrient transport. This potential is dependent on the net movement of nutrients across the plasma membrane (diffusion potential E_D) and on the activity of H^+-ATP-ases (proton pump) which moves the H^+ out of the cells increasing the positive charge of apoplast (the energy dependent component E_P). Both mechanisms form the resting membrane potential (E_M,) that is tissue and species specific, but is also relatively stable in certain conditions unless the cell membrane is damaged. In *Lotus corniculatus* cultivars, Al caused a strong decrease of E_D, but also affected E_P via decrease of activity of H^+-ATP-ases. Furthermore, this effect was in good correlation with a difference in sensitivity between cultivars (Pavlovkin et al., 2009). These changes in plasma membrane properties by Al affect its ion transport properties, mainly secondary active transport of nutrients.

2.3. Involvement of the oxidative stress

It is well known that Al treatment, directly or indirectly, initiates an oxidative burst; the rapid production of reactive oxygen species (ROS) such as hydroxide radicals, superoxide radicals and hydrogen peroxide. Accumulation of ROS in the cell can cause strong oxidative damage to cellular components. On the other hand, Al also induces expression of the genes encoding antioxidative enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidase (POD, EC 1.11.1.7), which may help in the removal of excess of ROS (Ezaki et al.,2000). The exact role of the ROS in Al toxicity is, however, still not fully understood. Some controversy is related to the fact, that increase of some certain ROS and/or antioxidant enzyme may be involved on oxidative signalling pathways (Foyer and Noctor 2005). A major kind of ROS in plants is hydrogen peroxide (H_2O_2) that acts as an important signal molecule involved in acclimatory signalling at low concentrations, whereas at high level can trigger programmed cell death. In wheat seedling, H_2O_2 pre-treatment improves wheat Al acclimation during subsequent Al exposure by enhancing the antioxidant defence capacity, which prevents ROS accumulation. That enhancement is greater in the Al-sensitive genotype than in the Al-tolerant one (Xu et al.,2011). Navascues et al (2012) found in relatively sensitive cultivar of *Lotus corniculatus* cv. INIA Draco, that treatment with low concentration of $AlCl_3$ (10 μM) was sufficient to inhibit root growth, but did not trigger ROS accumulation in root tips and also the expression of antioxidant enzymes was mostly unaffected. Therefore the authors conclude that the inhibitory effect of Al is unlikely the cause of oxidative stress in such conditions and also suggest that the accumulation of ROS, at high concentrations of Al, is rather a consequence not a cause of aluminium toxicity in the forage legume *Lotus corniculatus*. Among the antioxidant enzymes, an interesting change induced by Al is the replacement of the activity and protein level of the CuZnSODc by FeSODc (Navascués et al., 2012). The functional reason of such a change is unclear; more likely, the enzymes can compensate each other. It was previously found in *Arabidopsis,* that in Cu limited conditions, a micro RNA, *miR398,* mediates the down regulation of expression of both plastidic (CSD1) and cytosolic (CSD2) isoforms of CuZnSOD (Yamasaki et al.,2007). Thus the observed decrease of CuZnSOD in *Lotus* could be due to lower availability of Cu^{2+} (and maybe also Zn^{2+}) for the synthesis of the functional CuZnSOD protein.

2.4. Al-stress defence in Lotus

Many plant species evolved some mechanisms to improve their survival on acid soils, that can be divided into mechanisms that avoid entering of Al to cells (exclusion or resistance mechanisms) and those that would enable plants to accommodate Al^{3+} safely once it enter to symplast (tolerance mechanisms) (Ryan et al.,2011). The exclusion mechanisms include secretion of mucilage by the root border cells and exudation of organic acid anions from the root tip; the latter one being better understood (Inostrosa-Blancheteau et al 2012). Several research groups produced transgenic model lines in the last decade, increasing the synthesis of certain organic acids, or anion transport systems across the plasma membrane. Up to date, the largest success in increase of Al^{3+} avoidance has been achieved by over-expressing the organic anion transport proteins, the malate channels encoded by ALMT gene family (aluminium-activated malate transporter) and citrate secondary carriers encoded by MATE family (multidrog and toxic compound exudation), proving their important role in Al-resistance (Ryan et al 2011). A strong increase in ALMT mRNA levels was found in *Lotus corniculatus* after 14-d treatment with 20 μM $AlCl_3$, suggesting the involvement of malate (Navascues et al.,2012), although an increase of citrate and oxalate exudation was also observed when roots were exposed to high Al (0,5 mM) for short time (Pal'ove-Balang et al.,2012). Interestingly, an increase in fumarate exudation was also found in *Lotus japonicus* Gifu in the same conditions (Pal'ove-Balang et al., unpublished results). Therefore we can say that the involvement of organic acids in Al tolerance mechanism within the cell is generally possible, in spite of the fact that, in higher extent, it occurs mainly in Al -hyperaccumulator species (Ma et al., 2001).

In *Lotus*, metabolomic and HPLC data showed few changes in tissue organic acid content, the largest increase was found for threonic and 2-isopropylamic acid (Navascués at al. 2011). Therefore we think that Al-stress defences in *Lotus* must involve other mechanisms, in addition to organic acids. In a tolerant variety of *Lotus pedunculatus* (cv. Grasslands Maku) the formation of osmophilic Al-tannin complexes was found in vacuoles in the root apices. The ability of tannins to bind metals through complexation involving their *o*-diphenol groups was previously viewed as an negative effect impairing bioavailability of essencial micronutrients (Dixon et al.,2005). Stoutjesdijk et al., (2001) constructed a hypothesis, that the binding of Al by tannins and formation of Al-tannin complexes could remove toxic Al from the meristematic area and allows the growth of *Lotus pedunculatus* roots in Al-toxic conditions. In camphor tree (*Cinamomum canphora*), unique proanthocyanidin-accumulating cells were

identified in the root cap, shielding the rhizodermal cells (Osawa et al.,2011). Osmophilic tannin structures were also found in *Lotus corniculatus* root tips after Al treatment and were never present in acidic conditions without Al. The overall tannin content markedly increased after Al treatment in the root tips. However, no differences in tannin accumulation or formation of tannin structures in vacuoles were observed between two cultivars (INIA Draco and UFRGS) (see LOTASSA, 2010) that differed in their tolerance to Al.

The amino acid proline (Pro) is known to occur widely in higher plants and for a long time, it was considered as an inert compatible osmolyte that protects subcellular structures and macromolecules, under osmotic stress. Pro normally accumulates in response to several abiotic stress factors and recently has been assumed, that contributes to scavenging free radicals and stabilizing sub-cellular structures (Szabados and Savouvré 2010). After Al-treatment, proline accumulation occurs even at strong decrease of other free-amino acids and reduced N-assimilation in *Lotus* (Pal'ove-Balang and Mistrik 2011). In maize it has been shown that a tolerant variety accumulate more Pro than the sensitive one (Gianakoula 2008), but in *Lotus corniculatus* varieties this difference was not observed (Peter Paľove-Balang et al., unpublished results). The excessive amount of proline, if added externally or produced in transgenic *Arabidopsis* overexpressing pyrroline-5-carboxylate synthetase (P5CS), caused toxicity symptoms (Mattioli et al., 2008). The inhibitory effects of proline were found to be associated with the generation of reactive oxygen species, enhanced salicylic acid (SA) synthesis and PR genes expression. This suggested that proline could promote a reaction similar to hypersensitive response during pathogen infection (Chen et al., 2011). If this is true, then the possibility that proline could be involved in programmed cell dead in aluminum stress, resulting to the mosaic structure of cortical cell damage in the root tips (see 2.2.), can not be excluded.

In addition to mechanisms indicated above, in *Lotus japonicus* it was found an unusual phytochelatin synthase, LjPCS3, that is inducible by Fe and Al, unlike its analogues previously reported in *Arabidopsis* or *Glycine max* (Ramos et al., 2008). The authors have proposed a possibility that except for their function in heavy metal-detoxification PCS enzymes could play some complementary protective role also towards Al.

Figure 1 summarizes the different responses to Al observed in *Lotus* plants.

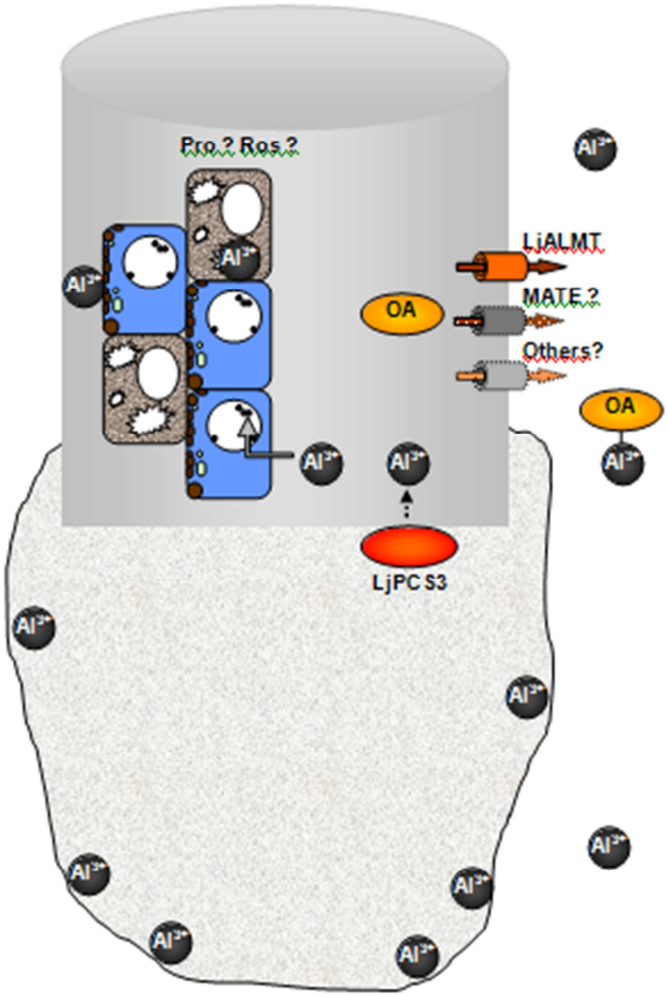


Figure 1. *Response of Lotus roots to Al toxicity*. Mucilage and border cells help to stop Al at the root tip. The most affected zone is the distal transition zone, where the cell division stops and cells are preparing for the elongation and differentiation. In this zone randomly distributed cortical cells are destructed (gray), whereas others are still alive (blue). Proline and ROS accumulation seems to be related to this selective cell dead. The alive cells produce cell wall protuberances (brown) and callose. Increased vacuolization occurs that often contains several electron dense inclusions that are likely tannin-Al complexes. Aluminum can be chelated by phytochelatine (LjPCS3 product, red) and organic acids. Chelation of Al with organic acids occurs mainly outside the roots, therefore Al-induced production of organic acid transporters such as LjALMT malate transporter (orange) is a key step. Citrate transporters of MATE family are also related to the resistance of some species (Ryan et al., 2011) and increased exudation of oxalate and fumarate suggests the involvement of other types of transporters.

3. DROUGHT STRESS

3.1. The importance of drought stress studies in Lotus

Various countries around the world experience drought in different ways but, in all cases, it leads to dramatic annual yield losses in crops and has

consistently detrimental physiological effects on the crop plants throughout (Bowne et al., 2012). One-third of the world's population resides in water-stressed regions, and with elevated CO_2 levels in the atmosphere and climatic changes predicted in the future, drought may become more frequent and severe in different regions (Dai, 2011). In different cropping systems around the world, drought is the main abiotic stressor, which is considered the most devastating and it is estimated to reduce yields drastically even under ideal growing conditions (Jogaiah et al., 2012). It has been observed that animal production in countries such as Uruguay is limited by the productivity and quality of natural pastures that represent more than 70 % of the grazing area. This was the reason why temperate forage legumes were adopted since the 60. Forage legumes are found to be important in the sustainability of agricultural and natural ecosystems with increments of up to 8-fold in the organic matter of agricultural rotations compared to monoculture systems. However, the low proportion of cultivated pastures reflects the difficulties in the establishment and persistence of introduced legumes (Rebuffo et al., 2007; LOTASSA, 2010). In the case of *Lotus*, rainfall distribution throughout the year determines important periods of water deficit, particularly during summer *L. corniculatus* yields 7.4-fold more in wet summers than in dry ones, a clear indication of the susceptibility to water restriction (Diaz Lago et al, 1996).

Current agriculture biotechnology's challenge is to satisfy increasing demand in food production. It is suggested that in the future, genetically modified crops should be developed with combination of desirable traits and introduction of new traits, particularly drought resistance, and this would be the case also for *Lotus* (Rebuffo et al., 2008). Drought resistance is a complex trait that involves morphological, physiological and biochemical changes. Recently, the omics tools for understanding stress tolerance are showing an upward trend, and, in the near future, a systems biology push is expected to provide a much needed impetus towards achieving a long-standing demand for better abiotic stress-tolerant food crops (Jogaiah et al., 2012). In the following sections we will summarize some of the recent data obtained from drought stress transcriptomics and metabolomics in Lotus.

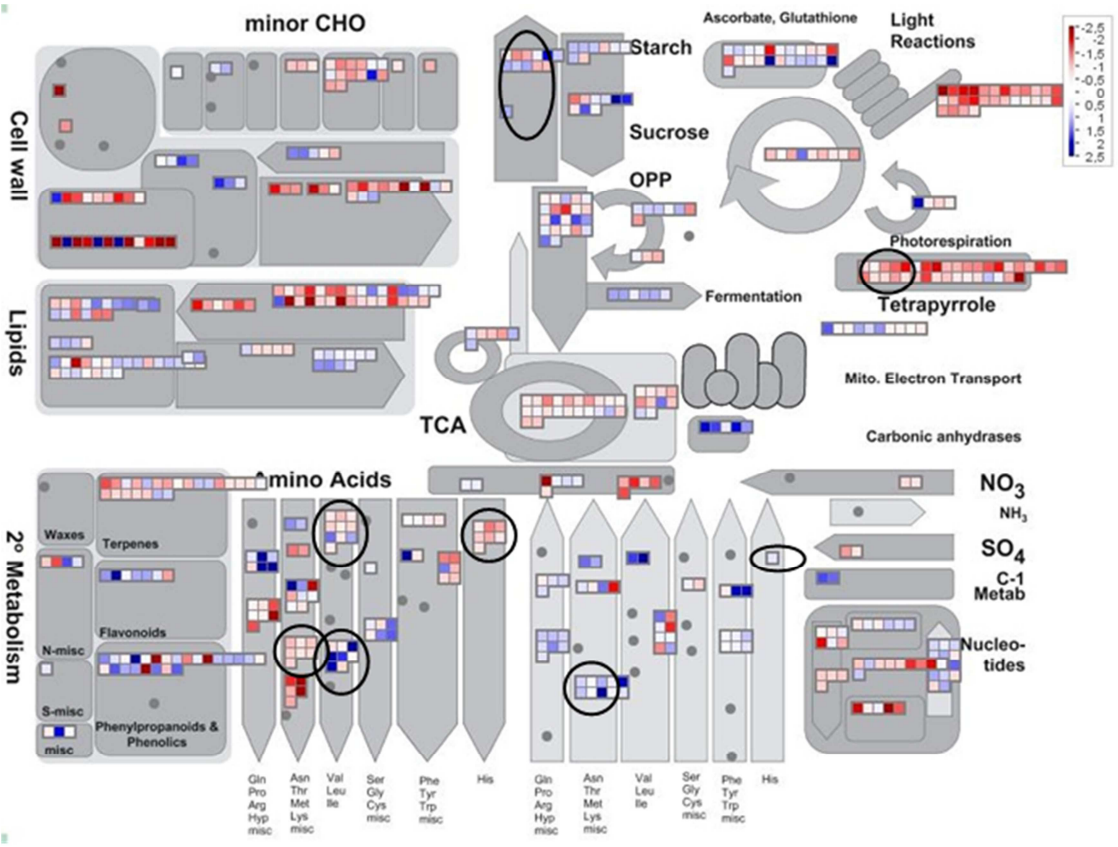
3.2. Lotus drought transcriptomics

The response to drought stress of the model legume *Lotus japonicus* has been recently studied using a transcriptomic approach. Drought induced an extensive reprogramming of the transcriptome which is related to various aspects of cellular metabolism and cellular stress response (Betti et al., 2012a). 3,950

probesets were shown to have altered levels of expression in response to drought. Fig. 2 shows a mapman overview of metabolism that highlights the metabolic pathways whose expression was more highly modulated according to the pathexpress tool ($p< 0.05$) (Goffard and Weiller, 2007). These pathways included starch and sucrose metabolism, branched amino acids (val/leu/ile) biosynthesis, lysine metabolism (biosynthesis and degradation), porphyrine and chlorophyll metabolism, and histidine metabolism (biosynthesis and degradation). Alkaloids biosynthesis and butanoate metabolism were also detected in pathexpress analysis (Betti et al., 2012), but do not appear in mapman Fig. 2.

The transcriptomic analysis carried out indicates that one of the major responses of *L. japonicus* plants to drought stress consists in a down-regulation of photosynthesis, since a high number of genes from porphirine and chlorophyll metabolism and light reactions were shown to be repressed under drought stress. Changes in photosynthesis in response to drought stress are common in different plant species (Saibo et al., 2009). Drought stress, like other kinds of abiotic stresses, induces stomatal closure, thus reducing the photosynthetic rate and affecting CO_2 assimilation and energy production (Chaves et al., 2003). This consequently results in the over-reduction of components within the electron transport chain that leads to the production of reactive oxygen species (ROS). The reduced expression of genes for photosynthesis may be then aimed to the reduction of ROS production. In *L. japonicus*, it has been recently shown that degradation of PSII could be caused by the loss of components of chloroplast antioxidant defence systems and subsequent decreased function of PS II (Sainz et al., 2010). In addition, changes in chlorophyll thermoluminiscence signals in response to drought were also detected in *L. japonicus* (Márquez et al., 2008), thus confirming important cahnges in the photosynthetic machinery of *Lotus* plants during drought. Considering that reduced photosynthesis levels should lead to lower energy and reduced carbon availability, it is easy to explain why it is also observed a modulation of starch and sucrose metabolism that may suggest remobilization of stored carbon reserves.

Figure 2. *Drought transcriptomics in Lotus.* The *Lotus* Affymetrix genechip was used containing 48,000 probesets for most genes known to be expressed in *L. japonicus* . This genechip was hybridized in triplicate with RNA extracted from leaves of five independent biological replicates of drought-stressed pots of plants (4 days water deprivation; relative water content around 65 %) and compared with the same results obtained for normally watered control plants. The number of genes whose expression was affected by drought was determined. 3959 probesets were found to be modulated by drought using a false discovery rate (FDR) of 5 % (FDR < 0,05). The figure shows a Mapman overview of metabolic pathways that were altered by drought . The most highly modulated pathways detected by pathexpress analysis are indicated in circles.



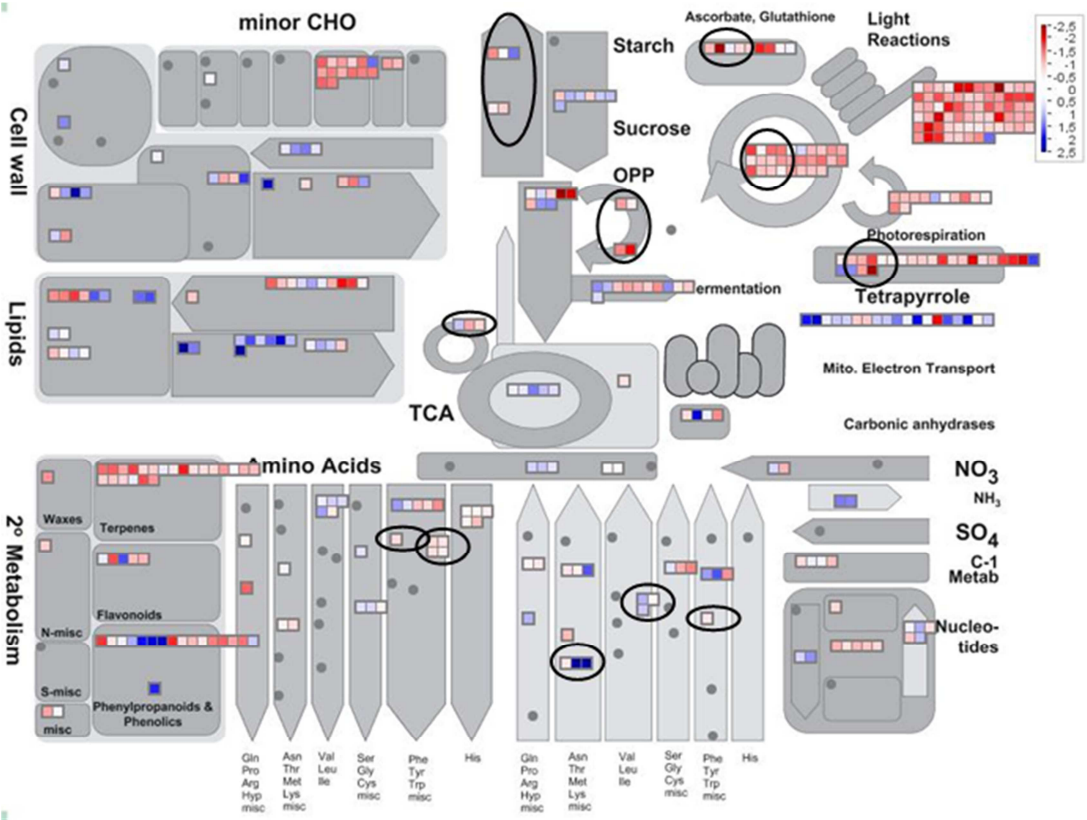
Changes in the transcriptome associated with amino acid metabolism (branched amino acids, lysine and histidine) may be another way to fuel de TCA cycle under drought conditions (Betti et al., 2012). The repression of the biosynthetic genes of the amino acids of the aspartate family (that includes lysine) and the concomitant induction of the corresponding catabolic genes is a general regulatory strategy in plant abiotic stress that cause energy deprivation (Galili, 2011). Recent results have also established a key role of N metabolism in the adaptation of plants to water stress (Yousfi et al., 2012).

The results from transcriptomic studies revealed also an ample number of genes belonging to different families of transcription factors that are highly responsive to drought in *L. japonicus* (Betti et al., 2012a). Several of them were homologues to known stress responsive genes from *Arabidopsis thaliana,* while some novel transcription factors were peculiar of the *L. japonicus* drought. Interestingly, recent results have shown an improvement of drought and salt tolerance in *Arabidopsis* and *L. corniculatus* by overexpression of a novel DREB transcription factor from *Populus euphatrica* (Zhou et al., 2012). This work can be given as an example of the enormous interest and potential of gene manipulation of transcription factors to improve drought stress tolerance in *Lotus*.

3.3. Drought transcriptomics in L. japonicus photorespiratory mutants deficient in plastidic glutamine synthetase (GS)

Ljgln2-2 mutants lacking plastidic GS (GS2) were recently used to determine that the lack of plastidic GS (GS2) produces four major consequences in response to drought in *L. japonicus* plants (Díaz et al.,2010; Betti et al., 2012b): (1) there is a compromised recovery (rehydration) of the plants following re-watering; (2) the level of proline accumulation by mutant plants is reduced under drought stress; (3) the pattern of expression of genes for proline metabolism is altered, producing a stimulation of the main (pyrroline-5-carboxylate synthase, P5CS) and alternative (arginase/ornithine-δ-aminotransferase) proline biosynthetic pathways, in parallel with a lack of induction of genes for proline degradation (pyrroline-5-carboxylate dehydrogenase); (4) massive changes in the transcriptome are produced. This means again a clear interconnection between nitrogen metabolism and the response to drought stress in *L. japonicus* plants.

We have further made use the pathexpress tool (Goffard and Weiller, 2007) to determine the most highly modulated metabolic pathways within the 2,304 probesets that showed differential levels of expression among wild type and *Ljgln2-2* mutant plants from *L. japonicus* in response to drought stress. The results obtained are shown in Figure 3. Several of the pathways obtained in this case corresponded again to phorphyrine and chlorophyll metabolism, starch and sucrose metabolism, as well as amino acid metabolism (lysine and brached amino acids degradations), butanoate metabolism and alkaloid biosynthesis, in a similar way than the core response to drought stress of *L. japonicus* wild type (WT) plants described above (see 3.2).



Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted	P-value	Adjusted p-value
Lysine degradation	map	12	11	8.41629004292674e-06	8.41629004292674e-06
Carbon fixation	map	22	14	0.000597225161785269	0.000597225161785269
Porphyrin and chlorophyll metabolism	map	20	12	0.00315930331897446	0.00315930331897446
Glycerolipid metabolism	map	18	11	0.00389977681467832	0.00389977681467832
Butanoate metabolism	map	21	12	0.00551353417184547	0.00551353417184547
Benzoate degradation via CoA ligation	map	15	9	0.0108729743280551	0.0108729743280551
Pentose and glucuronate interconversions	map	11	7	0.0166250761634233	0.0166250761634233
Glyoxylate and dicarboxylate metabolism	map	19	10	0.0233393835271931	0.0233393835271931
Tryptophan metabolism	map	19	10	0.0233393835271931	0.0233393835271931
Phenylalanine metabolism	map	14	8	0.0238028854044808	0.0238028854044808
Novobiocin biosynthesis	map	3	3	0.0238215506080608	0.0238215506080608
Nitrobenzene degradation	map	5	4	0.0262684530472894	0.0262684530472894
Starch and sucrose metabolism	map	31	14	0.0358469000724316	0.0358469000724316
Valine, leucine and isoleucine degradation	map	15	8	0.0385799266346817	0.0385799266346817
Alkaloid biosynthesis II	map	8	5	0.0486369443101993	0.0486369443101993
Ascorbate and aldarate metabolism	map	8	5	0.0486369443101993	0.0486369443101993
Tyrosine metabolism	map	24	11	0.0546280740766079	0.0546280740766079

Figure 3. *Drought transcriptomics in L. japonicus gln2-2 mutants deficient in plastidic GS.* The figure shows a Mapman overview of the metabolic pathways that were altered by drought in the mutant plants compared to the wild-type. The most highly modulated pathways detected by pathexpress analysis are indicated in circles. The bottom part of the figure shows the output from pathexpress analysis ($p < 0.05$). Other details as in Figure 2.

This is possibly due to the fact that 80 % of genes that responded to drought in WT did also respond in *Ljgln2-2* mutants but the vast majority of these genes changed more than 3 fold their levels of expression in the mutant compared to the WT. These data supported the idea that the mutant perceived or actually experienced higher cellular stress under drought than the wild-type (Díaz et al., 2010). However, the pathexpress analysis carried out also established other metabolic pathways that were significantly modulated ($p < 0.05$) in the

GS2-minus mutants such as: (1) glycerolipids metabolism and ascorbate metabolisms, which may be related to the higher oxidative stress and membrane damage postulated for mutant plants (Díaz et al., 2010); (2) carbon fixation, glyoxylate and dicarboxylate metabolisms, which are strongly decreased in the mutants and may correspond to a down-regulation strategy to avoid an excess of photorespiratory C2 metabolism which is impaired in the mutant plants (Orea et al., 2002; Márquez et al., 2005; Betti et al., 2006; Betti et al., 2012b; García-Calderón et al., 2012); (3) Tryptophan and phenylanine metabolisms, alterations that could be related with the changes observed in the expression of phenylpropanoids and phenolics secondary metabolism pathways in response to drought in the mutant plants.

3.4. Metabolite changes associated to Lotus drought

Proline is known to be involved in drought stress responses in *Lotus* for quite a long time (Borsani et al., 1999). A strong correlation was detected in *L. japonicus* between proline concentration and hydric deficit. Proline accumulation begins at early stages of drought imposition, when water content variations in the tissues are very low. As low as 10 % decreases in the relative water content are enough to trigger proline accumulation (Diaz et al., 2005d; LOTASSA, 2010). De novo proline accumulation in response to drought was also observed in all *Lotus* species examined so far, either model *(L. japonicus, L. burtii* and *L. filicaulis*) or other agronomic important species (*L. corniculatus, L. tenuis* and *L. uliginosus*) (LOTASSA, 2010; Diaz et al., 2005b). It has also been observed that proline content is affected by the kind of nitrogen source applied in the nutrient solution, since when *L. corniculatus* and *L. japonicus* plants are grown with ammonium, the proline accumulation is at least twice than in plants with nitrate (Díaz et al., 2005c; Díaz, 2011). On the other hand, the lower amount of proline observed in *L. japonicus* GS deficient mutants was correlated with a deffect in the rehydration ability of the mutant plants after drought (Díaz et al., 2010). Thus, proline accumulation was shown to be a clear marker of drought stress responses in *Lotus.*

Different sets of metabolomic experiments have been designed for further GC-MS metabolite profiling in order to determine the whole range of metabolites within different *Lotus* plant species that change under drought conditions. Different stress-dose-related metabolite changes were observed (Sánchez et al., 2012). Significant increases of organic acids, sugars and polyols were detected in *L. japonicus*. Organic acids included the TCA cycle intermediates succinic and

malic acid, while fructose, glucose, galactose and maltose, arabitol, ononitol and galactitol were among the most accumulated sugars and polyols respectively. As a chemical class, amino acids showed a variable response. Proline, as mentioned before, increased substantially, as well as leucine and isoleucine, while glutamate, serine, glycine and threonine decreased (Sánchez et al., 2012). The decrease in glutamate may be related to its use as precursor for proline biosynthesis. Decrease in serine and glycine, which are photorespiratory C2 cycle intermediates, may be related with a down-regulation of photorespiratory metabolism during drought. As for other *Lotus* species, the comparative metabolomic approach revealed conserved and unique metabolic responses to drought stress. Importantly, only few drought-responsive metabolites were conserved among all species (Sánchez et al., 2012). From a biotechnological perspective, these results highlight potential pitfalls of transgenic approaches towards improved crop tolerance when based on observations of the accumulation of specific compatible solutes. Even though translational approaches attempting to transfer the knowledge derived from a single model species or from the comparison of just one tolerant and one sensitive genotype are not necessarily compromised, there is a need to caution that the transfer of such results can be highly error prone (Sánchez et al., 2012).

4. CONCLUSION

Considerable progress has been made recently on the knowledge of *Lotus* responses to aluminum and drought conditions. Al-stress in *Lotus* produces important alterations in roots at the plant cell level, such as changes in vacuolar volume of young meristematic cells, formation of strong cell wall protuberances and callose depositions, as well as changes in plasma membrane ion transport properties. Oxidative stress, tannins, phytochelatins and proline are also important in Al-stress responses in *Lotus*. On the other hand, drought stress responses involve mainly changes in photosynthesis, oxidative stress and C-N metabolisms. Proline was shown to be a crucial amino acid both in Al- and drought-stress responses in *Lotus*. The new set of genetic resources available, such as model and cultivated species, ecotypes, mutant lines, genetic maps, RIL lines, etc. together with sequencing and functional genomics tools, have contributed significantly to the knowledge of abiotic stress in *Lotus* and make *Lotus* a very promising plant species for future research. Although the adaptive *Lotus* plants characteristics make them good candidates for restoration and phytoremediation of degraded environments, a deeper understanding of stress

responses is still needed to improve the adaptability and forage capabilities of *Lotus* cultivars.

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Publicación 5.

Cellular Stress Following Water Deprivation in the model legume *Lostus japonicus*.

Betti M, Pérez-Delgado C, García-Calderón M, Díaz P, Monza J, Márquez AJ (2012) *Cells* 1, 1089-1106.

ABSTRACT

Drought stress is one of the most important factors in the limitation of plant productivity worldwide. In order to cope with water deprivation, plants have adopted several strategies that produce major changes in gene expression. In this paper, the response to drought stress in the model legume *Lotus japonicus* was studied using a transcriptomic approach. Drought induced an extensive reprogramming of the transcriptome as related to various aspects of cellular metabolism, including genes involved in photosynthesis, amino acid metabolism and cell wall metabolism, among others. A particular focus was made on the genes involved in the cellular stress response. Key genes involved in the control of the cell cycle, antioxidant defense and stress signaling, were modulated as a consequence of water deprivation. Genes belonging to different families of transcription factors were also highly responsive to stress. Several of them were homologies to known stress-responsive genes from the model plant *Arabidopsis thaliana*, while some novel transcription factors were peculiar to the *L. japonicus* drought stress response.

1. INTRODUCTION

The study of the plant response to drought stress is very important. The alarming growth rate of the world's population, which depends mostly on plants for food energy intake, has led to an increased demand for crops with improved productivity. Drought stress, together with salinity, is one of the most important factors that reduces the yield of crops world-wide. Alongside classical breeding programs, functional genomics approaches are fundamental for the generation of plants with increased resistance to drought. A first step for the isolation of genes that may be related to drought tolerance is the identification of drought-responsive genes in the plant species considered. This can be now carried out thanks to the availability of DNA microarrays for several plant species. In this way, a great number of drought responsive genes have been identified, especially in the model plant *Arabidopsis thaliana* [1], and their contribution to drought or stress tolerance can be assessed in the laboratory. Genes that improve abiotic stress tolerance mostly encode for transcription factors, enzymes for the biosynthesis of sugars and compatible solutes, proteins of the antioxidant defense and ion transporters, among others.

Exposure to water shortage, especially when followed by rapid dehydration, triggers the induction of basic responses aimed to reduce water loss and the concomitant oxidative stress associated with it [2]. However, many more genes are induced during drought, as demonstrated by several transcriptomic studies. This includes the genes involved in stress sensing and signal transduction, together with several metabolic pathways that are modulated in order to maximize the fitness of the plant under water deprivation [1,3]. The vast number of genes that are modulated by water deprivation illustrates the severe stress conditions caused by drought at the cellular level. At the level of whole plant metabolism, severe drought causes inhibition of photosynthesis and a general metabolic dysfunction that compromises plant growth and fertility, and can lead to premature senescence [4]. Cellular responses to drought include the adjustment of the membrane system, which may be compromised under stress, as well as important changes in the cell cycle and cell division [4]. Several compounds and macromolecules are produced in order to deal with the water loss and the excess of reactive oxygen species produced. This includes chaperonins like heat-shock proteins and compatible solutes, and small molecules such as proline, glycine betaine and raffinose, which play several protective roles, for example, in helping to maintain cell turgor and scavenging reactive oxygen species (ROS) [1]. Other stress-responsive proteins produced under water deprivation include the late embryogenesis-abundant (LEA) proteins, which have a protective role during dehydration, aquaporins; these form pores in the lipid

bilayer and facilitate water flux and proteases, which are produced in order to get rid of damaged proteins and to remobilize nitrogen [1,5].

Among different plant species, the Leguminosae are second only to the Gramineae in importance to humans as a source of food, feed for livestock and as raw materials for industry [6]. The productivity of legumes can be hampered by drought stress, since this condition strongly limits nitrogen fixation in the nodules [7]. Unfortunately, cultivated legume species are poor models for genomic research. In fact, some of them are tetraploids and many have large genome sizes and are recalcitrant to transformation [7]. As a consequence, two legumes species, *Lotus japonicus* and *Medicago truncatula*, have been adopted internationally as models for legume research. In particular, *L. japonicus* serves as a model for the study of several other species of the genus Lotus that are highly used as pasture in temperate regions [8], where the plants can be exposed to sudden periods of drought. The response of *L. japonicus* to different kinds of abiotic stress has been studied at the transcriptomic, metabolomic and proteomic levels [9–12]. Several of these studies have been carried out thanks to the recent availability of an Affymetrix Genechip designed specifically for *L. japonicus*.

Previous work from our group demonstrated the important role played by the plastidic isoform of glutamine synthetase (GS) of *L. japonicus* in the response to drought stress and in drought-induced proline production [10,13]. These results were obtained by comparing the drought-stress transcriptomes of wild-type (WT) and mutant plants that lacked of plastidic GS. Since plastidic GS is fundamental for the reassimilation of the ammonium generated during photorespiration, mutants that lack of this enzyme show an air-sensitivity phenotype typical of plants that are impaired in one of the steps of the photorespiratory cycle [14,15]: plants can grow well under a CO_2-enriched atmosphere, where photorespiration is suppressed, but show several stress symptoms like chlorosis and necrosis when grown under normal air conditions. For this reason, previous transcriptomic studies that compared the response to drought of WT and plastidic GS mutants were carried out under CO_2-enriched atmosphere. In the present work we have studied the response of *L. japonicus* plants to drought stress under physiological conditions (normal air). The transcriptomes of well-watered and drought-stressed plants grown under normal air conditions were compared and, according to the aim of this special issue, a particular attention has been paid to the cellular mechanism of response to the stress conditions imposed by water deprivation.

2. RESULTS AND DISCUSSION

2.1. Drought Stress Transcriptomics of the Model Legume Lotus japonicus

In order to study the cellular response of *L. japonicus* plants to drought stress, a water deprivation experiment was carried out with 35 days-old plants. Drought was imposed by withholding watering for 4 days. After this period the plants showed a relative water content of about 60%. As demonstrated previously, this level of water loss does not compromise the performance of WT *L. japonicus* plants, which are still able to rapidly restore their water status if watered again [10]. Longer periods of water deprivation caused death of the youngest leaves and were not considered for this study. Leaves were harvested from drought-stressed plants and normally-watered plants, used as a control. The RNAs obtained were hybridized to the Lotus1a520343 Affymetrix Genechip®, which contains 52,749 unique probesets. A probeset is an oligonucleotide designed to measure the expression of a known or predicted sequence of mRNA. Several probesets may correspond to the same gene, in such a way that most of *L. japonicus* gene transcripts are analyzed in a single DNA chip. Drought-induced changes in the transcriptome were analyzed by a significance-based comparison of control and drought-stressed plants, applying a false discovery rate (FDR) of less than 0.05 and using three independent biological replicates for both control and drought-stressed plants. A validation of the microarray data was carried out by qRT-PCR. The expression levels of different genes for proline metabolism that are normally highly modulated by drought [10] were determined. A good agreement between qRT-PCR and microarray data was obtained (Figure 1).

2.2. Global Overview of the Dataset

In total, 3,950 genes were modulated after four days of water deprivation. The ratio between the number of induced and repressed genes was slightly biased towards induction, with 2,064 up-regulated and 1,886 down-regulated ones. The full list of the 3,950 genes that were significantly modulated by drought can be found online as supplemental material (Supplemental Table S1).

An overview of the different genes modulated by drought in relation to their correspondent metabolic pathways was generated using the MapMan program [16] (Figure 2). Many genes related to photosynthesis like the ones coding for the structural component of the photosystems (“Light Reactions” in Figure 2) and for the biosynthesis of photosynthetic pigments (tetrapyrroles) were repressed as a consequence of water deprivation, indicating that there is a general shutdown of photosynthetic metabolism in *L. japonicus* in response to drought.

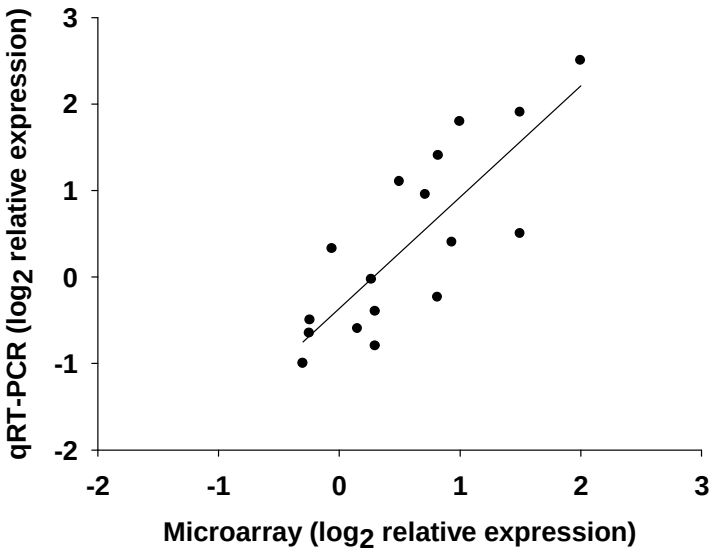

Figure 1. qRT-PCR validation of the microarray data. Each point represents one of the genes for proline metabolism that were previously used for the validation of Lotus microarray data [10]. The values reported in the graph are the $\log_2$ of the difference in expression levels between normally watered and drought-stressed plants. Linear regression analysis gave a regression coefficient of $r^2 = 0.67$. Values are the mean of three independent biological replicates.

The central carbon metabolism was also affected by drought conditions, with a general repression of the genes encoding for the enzymes of the TCA cycle. Modulation of both lipid biosynthesis and degradation was suggestive of reorganization of membrane composition and/or of membrane damage.

Consistent with these results was the increased level of lipid peroxidation previously observed in *L. japonicus* under drought conditions [10]. Several pathways for the biosynthesis and degradation of amino acids were also modulated by drought stress. Among them, genes encoding for pyrroline-5-carboxylate synthetase (P5CS) were induced (Supplemental Table S1). P5CS catalyzes the first step in the biosynthesis of proline, an amino acid that is normally accumulated in plant cells in response to different kinds of abiotic stresses [17]. The induction of other genes involved in the production of compatible solutes like trehalose and γ-aminobutyrric acid suggested an increased production of these molecules. A gene encoding for trehalose-6-phosphate synthase was induced about two-folds (probeset Ljwgs_070708.1; Supplemental Table S1).

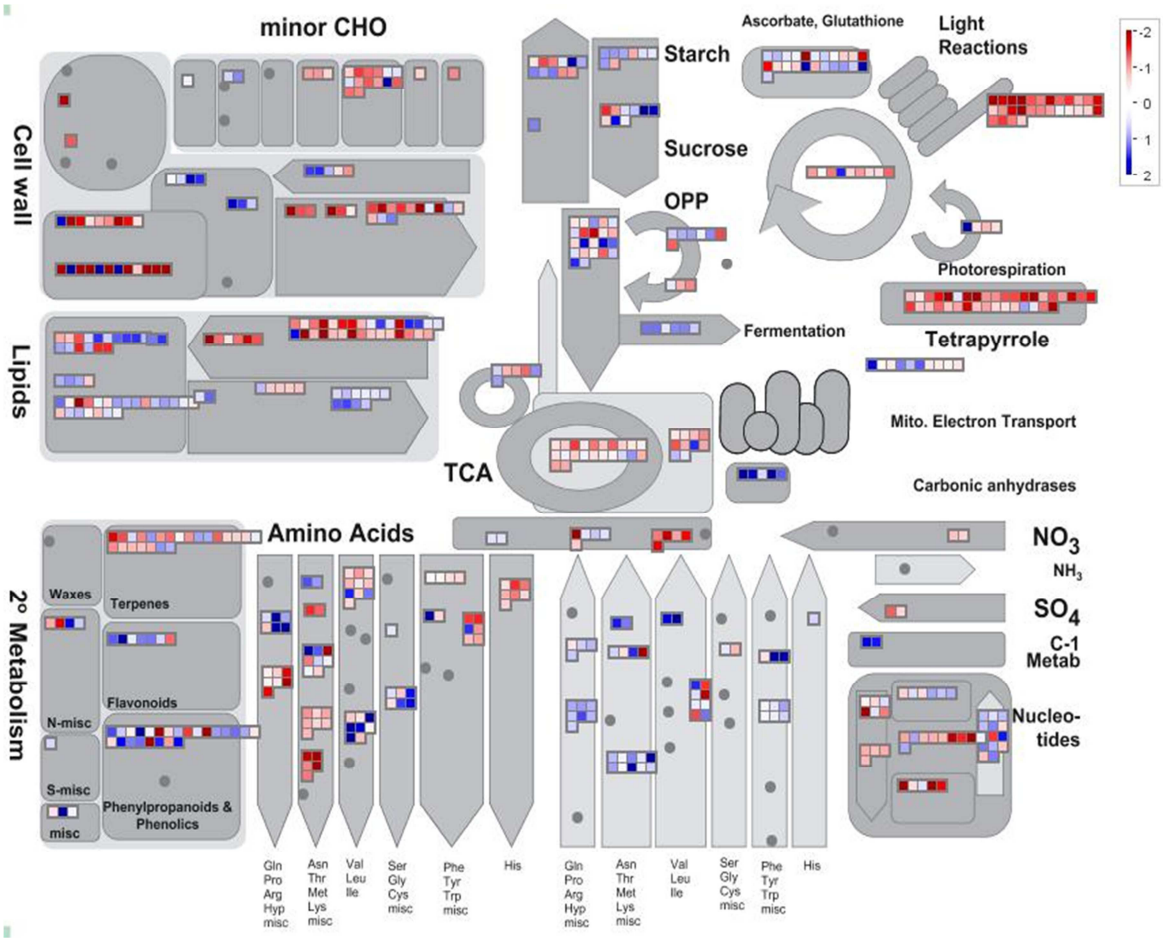

Figure 2. MapMan general metabolism overview of the genes modulated by four days of drought in *L. japonicus* plants. Each square corresponds to a gene. Red and blue indicate lower and higher expression than the control, respectively. The scale bar is shown in $\log_2$.

Interestingly, overexpression of this gene in tobacco plants lead to increased drought tolerance [18]. The data obtained indicate that *L. japonicus* plants undergo an extensive reprogramming of the transcriptome in response to drought stress. Considering the great number of genes and pathways affected by water deprivation, a further analysis of the dataset was carried out focusing on the identification of the genes and metabolic pathways that were most significantly modulated.

2.3. Analysis of the Most Modulated Genes

Highly stress-responsive genes are good candidates for the evaluation of their contribution to drought tolerance in targeted studies, either by overexpression of the candidate gene or by the obtention of specific mutants [1]. In the case of *L. japonicus*, the development of a TILLING reverse genetic tool

[19] and, much more recently, of a population of insertion mutants created using the LORE1 endogenous retrotransposon [20] allow the rapid obtention of mutants in a selected gene. For this reason, we focused our analysis on the top 10 up- and down-regulated genes in the drought-stress transcriptome (Table 1). The gene sequences were blasted against the current databases and the TAIR database [21] and the corresponding Arabidopsis orthologous genes were identified.

The most drought-induced gene is ortholog to the Arabidopsis 16 kDa outer plastid envelope protein Oep16. This gene was also among the most induced by drought under CO_2-enriched atmosphere [13]. The corresponding protein product belongs to a family of pre-protein and amino acid transporters present in chloroplasts and mitochondria of plants, as well as in bacteria [22]. Proteins of the mitochondrial and plastidic protein import machineries are often modulated by different kinds of abiotic stresses [23]. Considering the limited protein encoding capacity of these organelles, it is easy to understand that many of the protein and enzymes required in response to stress depend on the import of cytosol-synthesized proteins. This may explain the high induction observed for the *L. japonicus* Oep16 ortholog. The second and third most induced genes belong to the LEA family (probesets Ljwgs_133863.1 and chr1.TM0221.11). LEA genes encode for a broad group of stress-protection proteins that are expressed during embryo maturation in several plants [1], whose precise biochemical way of action is still not fully understood [5].

Genes involved in the antioxidant response like a glutathione-S-transferase (probeset Ljwgs_074013.2) and a nucleoredoxin (probeset Ljwgs_026189.1) were also highly induced by drought, suggesting increased oxidative stress. Nucleoredoxin are multi-domain thioredoxins, whose function remains still rather unexplored in plants [24], while glutathione-S-transferases are involved in xenobiotics detoxification, ROS scavenging and may remove peroxidized lipids [25]. A gene encoding for beta glucosidase, a protein that hydrolyzes glycosides of abscisic acid (ABA) to liberate active ABA, was highly induced (probeset chr2.CM0056.64). ABA is important in the response to drought stress since it causes stomatal closure, which prevents excessive water loss and induces the expression of stress-related genes [26]. It is worth noting that overexpression of beta glucosidase in *Arabidopsis* resulted in increased drought and salt tolerance [27]. Different genes encoding for transcription factors (TFs) were also highly modulated (Table 1). Interestingly, while a NAM, ATAF1/2 and CUC2 (NAC) domain TF ortholog to Arabidopsis NAC47 was highly induced (probeset chr1.CM0104.32), RAD-like 5 (AtRL5), a gene related to the myeloblastosis (MYB) family of TFs was the most repressed under drought stress (probeset

chr2.CM0249.113). RAD-like transcription factors are a subfamily of the MYB factors. Members of the RAD-like family of TFs are involved in floral development in Arabidopsis [28]. However, the exact role of AtRL5 is unknown. On the other hand, NAC47 was described in Arabidopsis as a gene responsive to ammonium supply in a previous transcriptomic study [29].

Among the most drought-repressed genes there was one encoding for expansin, an enzyme involved in cell-wall loosening during the enlargement of plant cells. This may indicate that the cells are undergoing cell wall restructuration under water deprivation. Other highly repressed genes encoded for proteins of the aspartyl protease family (probesets Ljwgs_065092.1 and chr6.CM0139.42), a chaperon protein (probeset chr3.CM0112.48) and two transposable elements (probesets BM0976.11 and Ljwgs_098953.1). A novel NH_4^+ transporter of the LjAMT1 family (probeset Ljwgs_028040.1), with 89% similarity to LeAMT1.3 from tomato was repressed more than 20-fold ($2^{4.42}$). This transporter was also highly repressed by salt stress [9] and by drought under CO_2-enriched atmosphere [13].

Another repressed gene related to ammonium transport (probeset chr3.TM0745.32) was homolog to the Arabidopsis delta tonoplast integral protein AtTIP2;1. This protein is involved in ammonium transport into the vacuole and the corresponding gene is induced by ammonium [30]. Further studies should be needed in order to understand why several genes related to ammonium transport are modulated in *L. japonicus* under abiotic stress conditions.

In summary, these results indicate that the most regulated genes in *L. japonicus* cells under water deprivation are involved in several aspects of cellular metabolism, including the production of protective molecules, oxidative stress response, transport, cell wall restructuration, transcription and hormone metabolism among others. Some of these processes are related to general cellular stress responses such as the deformation and damaging of membranes, lipids, proteins and DNA together with the generation of oxidative stress [31]. On the other hand, processes like cell wall restructuration and transport of water and ammonium are probably more specific to the response to drought stress.

Probeset	log_2 FC	Arabidopsis ortholog	Locus
Up-Regulated			
chr4.CM0429.5	4.57	Outer plastid envelope protein Oep16	At4g16160
Ljwgs_133863.1	4.53	LEA7	At1g52690
chr1.TM0221.11	4.24	LEA4-5	At5g06760
Ljwgs_062789.1	4.16	oxidoreductase	At5g09300
chr2.CM0056.64	4.01	Beta-glucosidase	At1g02850
Ljwgs_013141.2	3.94	Putative protein	At2g25625
Ljwgs_053770.1	3.82	Putative protein	At5g66780
Ljwgs_074013.2	3.71	Glutathione-S-transferase	At2g29490
chr1.CM0104.32	3.70	NAC47	At3g04070
Ljwgs_026189.1	3.66	Putative nucleoredoxin	At1g60420
Down-regulated			
chr2.CM0249.113	−5.14	AtRL-5	At1g19510
Ljwgs_015206.1	−4.70	Expansin	At1g26770
BM0976.11	−4.46	Retrotransposon	n.d.
Ljwgs_065092.1	−4.44	Aspartyl protease family protein	At1g03220
Ljwgs_028040.1	−4.42	AMT1;4	At4g28700
chr3.TM0745.32	−4.34	Delta tonoplast integral protein AtTIP2;1	At3g16240
chr6.CM0139.42	−4.18	Aspartyl protease family protein	At1g03220
chr3.CM0112.48	−4.16	DNAJ-like chaperone	At4g36040
Ljwgs_098953.1	−4.14	Retrotransposon	At4g27210
chr1.CM0233.42	−3.94	Transposable element	At1g35920

Table 1. Top 10 genes up- or down-regulated by drought in leaves of *L. japonicus*. The fold-change (FC) is expressed as the log_2 of the difference in relative expression levels between drought stress conditions and normal watering. The description and locus identifier of the Arabidopsis orthologous genes are also reported.

2.4. Analysis of Overrepresented Pathways

The effect of drought on the expression of different functional groups of genes was tested. The percentage of the total number of genes modulated by drought within each functional category is indicated in Figure 3. 16 of the 36 functional groups defined by the MapMan software showed a modulation of at least 10% of their total genes, confirming that drought induces an extensive reprogramming of the transcriptome. Six functional categories showed changes in the expression of more than 20% of their members: tetrapyrrole synthesis

(where almost the 45% of the genes were modulated), gluconeogenesis/glyoxylate cycle, amino acid metabolism, TCA cycle, nucleotide metabolism and redox regulation.

In order to determine if the high modulation of these metabolic pathways was statistically significant, the dataset was analyzed using the program Pathexpress [32]. This algorithm allows the identification of the most relevant metabolic pathways within a group of genes. Using a P cutoff value of less than 0.05, the program identified eight over-represented pathways (Figure 4). First of all, the analysis carried out with Pathexpress confirmed that the biosynthesis of photosynthetic pigments was highly repressed under drought conditions. This down-regulation of photosynthetic metabolism observed is a common response to high levels of stress [33] and may suggest a decrease in photosynthesis in *L. japonicus* under drought. The other over-represented metabolic routes felt mainly under the categories of carbon and amino acids metabolism, in good agreement with the data presented in Figure 3. Of particular interest was the fact that the pathways for both lysine biosynthesis and degradation were highly regulated. Several genes for lysine biosynthesis were repressed, while genes for lysine degradation were induced, suggesting a decrease in the lysine pool as a consequence of drought. The repression of the biosynthetic genes of the amino acids of the aspartate family (that includes lysine) and the concomitant induction of the corresponding catabolic genes is a general regulatory strategy observed in plant abiotic stresses that cause energy deprivation [34]. Under such conditions, lysine degradation may contribute to cellular energy metabolism by providing carbon skeletons to fuel the TCA cycle [35].

Taken together, these results indicate that the metabolic pathways that are more regulated by drought stress in *L. japonicus* are related to carbon and amino acid metabolisms. Drought stress, like other kind of abiotic stresses, induces stomatal closure, which reduces the photosynthetic rate and affects the rate of CO_2 assimilation and energy production [2]. This, consequently, results in the over-reduction of components within the photosynthetic electron transport chain that leads to the production of ROS. The reduced expression of genes for the biosynthesis of photosynthetic pigments may then be aimed to the reduction of ROS production through a reduced activity of the components of the photosystems. On the other hand, reduced photosynthesis levels should lead to lower energy and reduced carbon availability. The modulation of starch and sucrose pathways (Figure 4) may suggest remobilization of stored carbon reserves. This, together with the degradation of amino acids, may serve to fuel the TCA cycle under drought conditions.

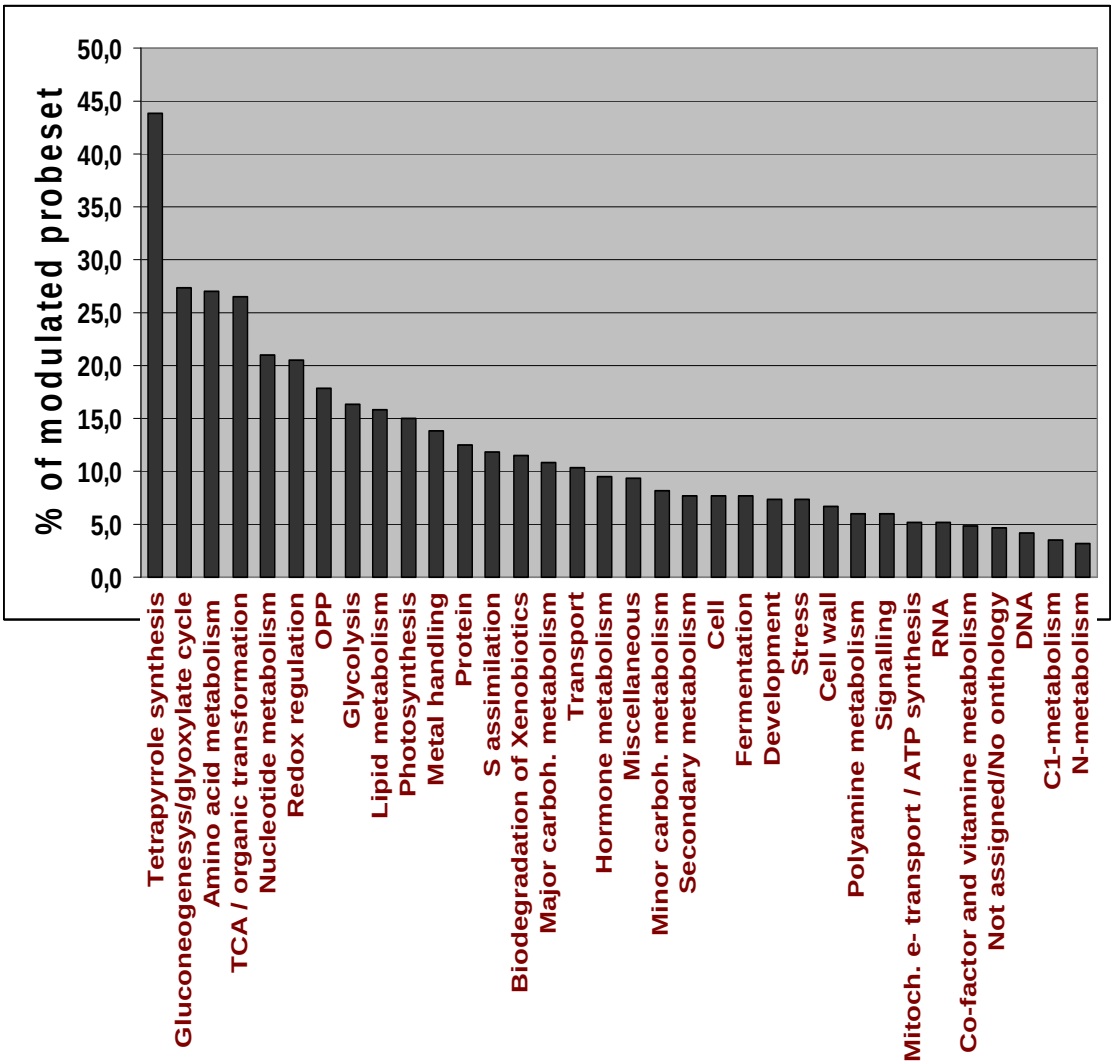

Figure 3. Percentage of transcripts from the 36 functional groups (or BINs) defined by the MapMan software that were significantly modulated by drought. The functional category "unassigned" was not considered in this analysis.

Pathway Name		Nb. of Enzymes	Nb. of Enzymes submitted	P-value
Starch and sucrose metabolism	map	31	21	0.00702267142913549
Valine, leucine and isoleucine biosynthesis	map	12	10	0.00703765641220921
Lysine biosynthesis	map	11	9	0.0134952392471356
Alkaloid biosynthesis II	map	8	7	0.0169136515689419
Porphyrin and chlorophyll metabolism	map	20	14	0.0186783782815911
Lysine degradation	map	12	9	0.0328679877519147
Butanoate metabolism	map	21	14	0.0336016682767735
Histidine metabolism	map	16	11	0.0444701950201206
Pyrimidine metabolism	map	22	14	0.0555414483123045
Pyruvate metabolism	map	26	16	0.06001338338036

Figure 4. Analysis of the dataset using Pathexpress. The significantly ($p < 0.05$) over-represented pathways are highlighted in red.

2.5. Overview of the Cellular Response to Drought Stress in Lotus japonicus

The modulation of genes involved in central metabolism and in the production of defensive molecules previously described was not the only component of the cellular response to drought. In fact, a great number of genes are involved in the perception of the stress and in the consequent transmission of the stimuli to the nucleus [26]. This is usually initiated by the activation of signaling cascades that comprehend protein kinases, calcium, phospholipids, hormones and transcription factors [1]. One of the signals that triggers these signaling cascades under different stress conditions is the production of ROS. Plant cells have developed a number of strategies in order to cope with these toxic molecules [25]. In this section, we will analyze the cellular response of *L. japonicus* to drought with a special focus on the genes encoding for antioxidant enzymes and transcription factors.

Surprisingly, the cellular response to water deprivation included the modulation of several genes involved in the perception and response to other kinds of stresses in addition to drought (Figure 5). Several genes classified as responsive to biotic stress, heat, cold and wounding were recognized by the MapMan software among the modulated ones. This may be explained by the fact that the transcriptomic responses to different kinds of abiotic stresses partially overlap [36]. In addition, both biotic and abiotic stresses are also known to regulate overlapping groups of genes [37]. This is probably due to the fact that ROS, which are generated under biotic and abiotic stress, are a common signal that triggers downstream stress responses [37]. Consistent with this hypothesis is the fact that several known and unknown genes of the *L. japonicus* redox defense were regulated under drought conditions (Figure 5). Previously described redox genes that were modulated by drought included several genes coding for isoforms of glutathione peroxidase like *LjGPX1*; *LjGPX2* and *LjGPX3* (probesets chr4.CM0558.29.1, chr4.CM0558.28 and Ljwgs_038927.1 respectively) [38]. Interestingly, the expression of these three isoforms of glutathione peroxidase was not induced by salinity and was repressed by toxic metals like Cd in *L. japonicus* [38]. Other known redox genes modulated by drought were the plastidic iron superoxide dismutase (LjFeSOD1, probeset gi46402889) [39] and different isoforms of thioredoxin and peroxiredoxin [40].

The expression of several genes involved in the control of cell cycle, cell division and plant development was also altered under drought conditions (Figure 5). Several cyclins, as well as mitotic control proteins and proteins involved in cell division were present among these two groups. This is compatible with an

arrest in plant growth and a decrease in the rate of cell division, both common responses of plants to drought or salinity [1].

An overview of the transcription factors (TFs) that responded to drought stress is presented in Figure 6. Members of several TF families that play a pivotal role in the response to drought were highly regulated, including basic leucine zipper (bZIP) domain TFs, zinc finger proteins like the basic helix-loop-helix (bHLH) family, MYB and MYB-related proteins and NAC domain TFs. Moreover, water deprivation triggered the coordinate repression of genes involved in the regulation of DNA structure and functionality like several genes encoding for histone proteins and DNA methyltransferases (DNA MT, Figure 6). This may indicate a reduced cellular division in *L. japonicus* under drought stress, as also suggested by the modulation of genes encoding for cyclins previously described (Figure 5). The genes encoding for the most modulated TFs in response to drought stress were further analyzed (Table 2). Since legume TFs and particularly *L. japonicus* are still poorly characterized [6], the Arabidopsis orthologs to the most modulated TFs were identified. About 250 TF were identified in *L. japonicus* by the MapMan program (Figure 6). For this reason, a four-fold change threshold was applied ($\log_2$ of fold change > 2 or < −2) in order to consider only the most modulated genes. The identification of highly drought-responsive transcription factors is of particular interest since they may represent good candidates for the engineering of plants for improved stress resistance. Moreover, a further analysis of this group of genes was carried out by comparing the data presented here with a previously reported transcriptomic study carried out with the plastidic GS mutant *Ljgln2-2* under drought conditions [10]. Since plastidic GS is involved in the stress responsive machinery of *L. japonicus* [10], it was interesting also to determine if the same or different transcription factors were involved in the response to drought in the mutant background.

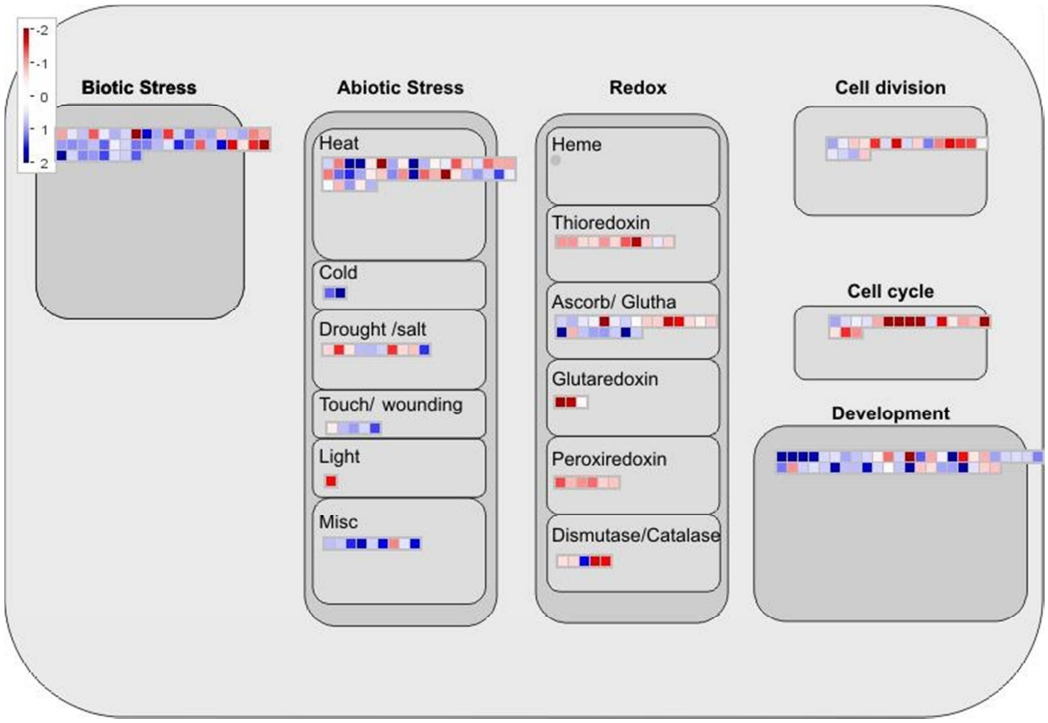

Figure 5. MapMan overview of the cellular response of *L. japonicus* to drought stress. Other details as described above.

The TF family that showed more highly induced genes was NAC, with four members induced more than four-fold in the WT (Table 2). The Arabidopsis orthologs to these NAC TFs were the previously described NAC 47 (Table 1), the NAC domain protein responsive to desiccation 26 (RD26), NAC100 and NAC1. Among these genes, only RD26 has been described as responsive to abiotic stress in Arabidopsis [41].

It is possible that some of these NAC genes are involved in the response to drought stress specifically in *L. japonicus*. Two highly induced genes were related to abscisic acid (ABA), an hormone that plays a central role in the response to drought and salinity [26]: MYB96, that regulates drought stress response by integrating ABA and auxin signals in *Arabidopsis* [42], and Aba repressor 1 ABR1 [43], a repressor of ABA-regulated gene expression. Both TFs are important for stress tolerance in Arabidopsis since overexpression of MYB96 resulted in enhanced drought resistance [42] and ABR1 mutants were hypersensitive to drought as well as other kinds of abiotic stresses [43].

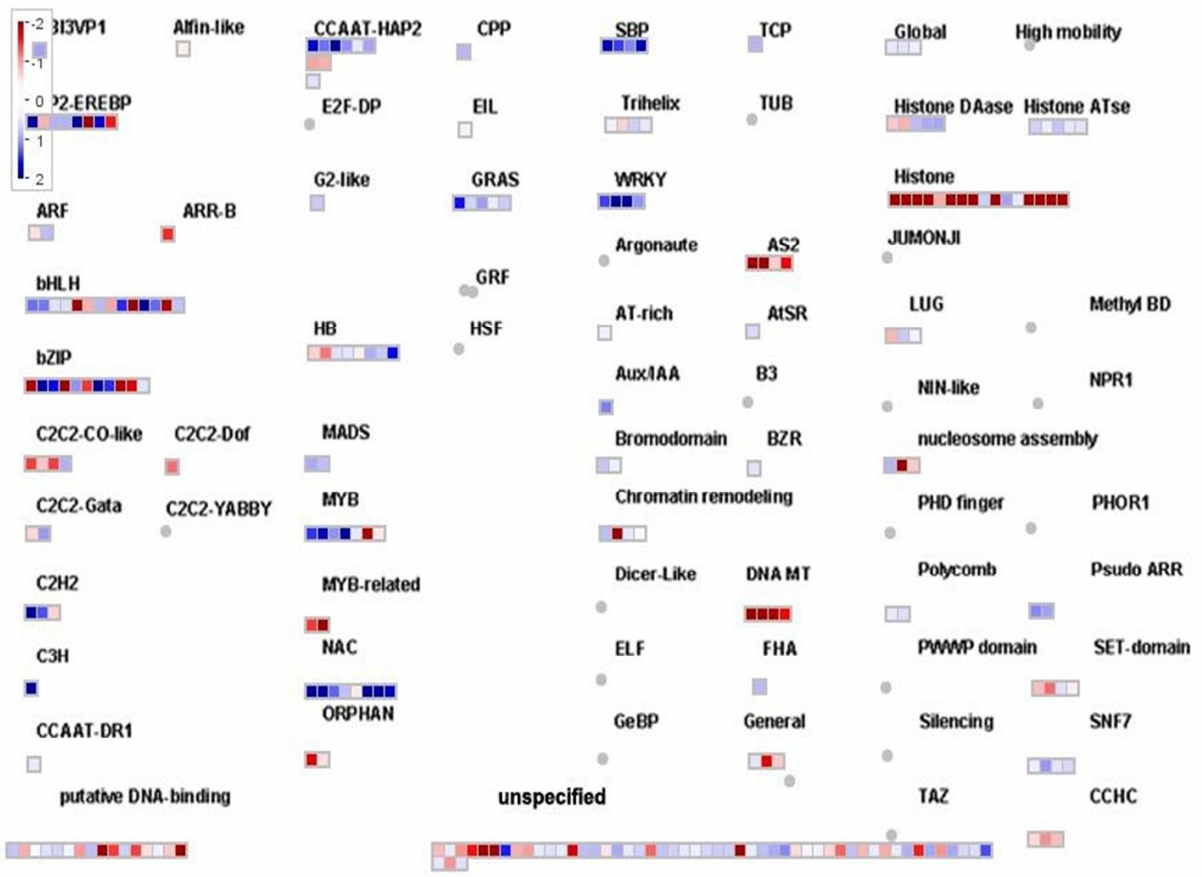

Figure 6. MapMan overview of the transcription factors genes that were modulated under drought conditions. Other details as described above.

These data suggest that the ABA signaling pathway under drought conditions is at least in part conserved between Lotus and Arabidopsis. Other induced genes with known Arabidopsis orthologs included the ethylene responsive transcription factor RAP2.10, AtOZF1 (oxidation related zinc finger), a protein related to oxidative stress tolerance [44] and STZ (salt tolerance zinc finger), a TF implied in the response to salt stress [45].

Probeset	log_2 FC		Arabidopsis ortholog	Locus
	WT	*Ljgln2-2*		
Up-Regulated				
chr1.cm0104.32	3.70	5.37	NAC47	At3g04070
Ljwgs_036303.1	3.26	5.68	NAC domain RD26 TF	At4g27410
chr5.cm0052.19	3.01	5.95	ABR1, AP2 domain TF	At5g64750
chr5.cm0040.40.1	2.71	4.10	WRKY40	At1g80840
chr1.cm0023.10	2.66	n.s.	MYB96	At5g62470
chr3.cm0724.4	2.46	3.22	CCAAT box AtHAP2C	At1g72830
Ljwgs_031732.1	2.42	4.19	bZIP-1	At1g77920
Ljwgs_134387.1	2.16	n.s.	RAP2.10	At4g36900
chr4.cm0087.91	2.09	3.31	NAC100	At5g61430
chr4.cm0004.18	2.04	n.s.	AtOZF1	At2g19810
chr5.TM1598.10	2.02	n.s.	bHLH family TF	At1g09250
chr5.cm0200.109	2.01	3.91	STZ, ZAT10	At1g27730
chr3.cm0279.50	2.00	n.s.	AtNAC1	At1g56010
Down-regulated				
chr2.cm0249.113	−5.14	−7.27	AtRL-5	At1g19510
Ljwgs_140411.1	−2.78	−3.34	bHLH family TF	At1g72210
chr4.cm0128.29	−2.76	−4.05	AtLBD37	At5g67420
Ljwgs_049909.2	−2.59	−3.44	AtLBD38	At3g49940
Ljwgs_020020.1	−2.57	n.s.	AtPCNA2	At2g29570
chr1.cm0178.64	−2.27	−3.57	CIB1	At4g34530
chr6.cm0082.29	−2.19	n.s.	AtBARD1	At1g04020
Ljwgs_141699.1	−2.01	−2.36	ArERF72	At3g16770
Ljwgs_032996.1	−2.00	−3.59	AtBZIP10	At4g02640

Table 2. Highly drought-modulated genes encoding for transcription factors. The fold-change (FC) is expressed as the log_2 of the difference in relative expression levels between drought stress conditions and normal watering. Transcriptional data for the plastidic GS mutant *Ljgln2-2* are from Díaz *et al.* [10]. The WT and mutant plants used for this analysis showed similar levels of water loss (relative water content of 60.0% ± 2.5%). n.s.: not significant.

The most repressed TF gene was ortholog to AtRL-5, whose function in Arabidopsis is unknown as mentioned previously (Table 1 and relative discussion). Two different members of the lateral organ boundary domain (LBD) family were repressed under drought conditions and corresponded to Arabidopsis AtLBD37 and AtLBD38. These two TFs are involved in the repression of anthocyanin biosynthesis and are important components in plant NO_3^-/N signaling [46]. The other down-regulated TFs also did not have any known role in response to drought. Interestingly, two highly repressed genes were orthologs to Arabidopsis ones involved in DNA repair: AtPCNA2 (proliferating cell nuclear antigen 2) [47] and AtBARD1 (breast cancer associated ring 1), that is also involved in stem cells development in the shoot apical meristem [48]. Other repressed TFs included orthologs to a basic helix-loop-helix (bHLH) TF with

unknown function (probeset Ljwgs_140411.1), a cryptochrome interacting basic helix-loop-helix (CIB) transcription factor, a basic leucine zipper (AtBZIP10) and the ethylene response factor ERF72. The analysis presented indicates that while several highly induced genes encoding for TFs are part of the known response to abiotic stress, the down-regulated ones seem involved in several cellular processes apparently not related to stress. On the other hand, Table 2 also shows that the majority of the TF genes modulated in the WT plants under water deprivation were also modulated in the plastidic GS mutant *Ljgln2-2*. This indicates that the modulation of TF genes transcription in *L. japonicus* in response to drought is not dependent from the presence of plastidic GS. This is of particular interest since the *Ljgln2-2* mutant showed a peculiar response to recovery after drought, and an about three times higher number of genes were regulated in response to drought in this genotype compared to the WT [10]. It is also worth noticing that the mutant presented a higher extent of modulation of all the TF genes considered that were significantly regulated in both genotypes (Table 2). This probably reflects the higher level of stress that is received by the mutant at the same level of hydric deficit than the WT [10].

3. EXPERIMENTAL

3.1. Plant Growth and Drought Treatments

L. japonicus (Regel) Larsen cv, Gifu seeds were initially obtained from Prof. Jens Stougaard (Aarhus University, Denmark) and then self-propagated at the University of Seville. The seeds were scarified and surface-sterilized, germinated in 1% agar Petri dishes, and transferred to pots using a 1:1 (v/v) mixture of vermiculite and sand as solid support. Five seedlings were planted in each pot and grown in a growth chamber under 16/8 h day/night, 20/18 °C, with a photosynthetic photon flux density of 250 μmol/m^2·s and a constant humidity of 70%. Non-nodulated plants were watered with Hornum nutrient solution, containing 5 mM NH_4NO_3 and 3 mM KNO_3 [8]. Drought conditions were applied by withholding irrigation to 35 days old plants. At this stage the plants had an average of seven fully expanded trifoils. The relative water content (RWC) of the leaves and the soil was measured each day. After 4 days of water deprivation the leaves were harvested, flash-frozen in liquid nitrogen and stored at −80 °C until use. The average RWC of the plants after four days of water deprivation was of 60.0% ± 2.5%, while the vermiculite/sand support used as soil showed a RWC of 31% ± 3.0%.

3.2. Measurement of Leaf and Soil Water Content

The water status of the leaves was expressed as the relative water content (RWC), calculated from the fresh weight (FW), dry weight (DW) and turgid weight (TW) of detached trefoils as follows: RWC (%) = 100 × (FW − DW)/(TW − DW). Dry weight was measured after incubation of the tissue overnight at 80 °C. Turgid weight was obtained after incubation of the detached trefoil for 8 h in water in a closed Petri dish.

Soil RWC was defined as: RWC (%) = 100 × (FW − DW)/(SW − DW) where FW, DW and SW refer to soil fresh weight, oven-dry weight and weight at field capacity respectively.

3.3. RNA Extraction, Genechip Hybridization and qRT-PCR

Leaf material was immediately frozen in liquid nitrogen after harvest, homogenized with mortar and pestle and kept at –80 °C until use. Three independent biological replicates were used for qRT-PCR and transcriptomic analysis. A biological replicate consisted of a pool of tissue from five plants that were grown in the same pot. Total RNA was isolated using the hot borate method [9]. The integrity and concentration of the RNA preparations was checked using an Experion bioanalyzer (Bio-Rad) with RNA StdSens chips and a Nano-Drop ND-1000 (Nano-Drop Technologies) respectively. RNA samples were labeled using the One-Cycle Target labeling Kit (Affymetrix), hybridized to the Affymetrix Genechip® Lotus1a520343 and scanned according to the manufacturer's instruction. MIAME compliant data were deposited at Array Express [49] as E-MEXP-3710. qRT-PCR validation of the microarray data using genes for proline metabolism was carried out as previously described [10].

4. CONCLUSIONS

In this paper we have shown that water deprivation induced an extensive reprogramming of the transcriptome in *L. japonicus*. This included several cellular processes such as the production of protective molecules, oxidative stress response, transport, cell wall restructuration, transcription and hormone metabolism, among others. The metabolic pathways that were significantly more regulated under drought conditions were related to carbon and amino acid metabolism. The transcriptional modulation of several genes involved in the control of the cell cycle was probably aimed to stop cell division and plant growth. Several highly drought responsive transcription factors were identified. Some of these genes were orthologs to Arabidopsis ones, with an important role in the response to abiotic stress, while others were probably peculiar to the Lotus

drought stress response. Further experiments should be designed with the aim of characterizing these novel genes and the assessment of their eventual contribution to drought tolerance. The data set presented here also contributes to the global characterization of gene regulation in Lotus, a topic of great interest, recently approached for other purposes [50,51].

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OTRAS PUBLICACIONES

Publicación 6.

Glutamine synthetase in legumes: recent advances in enzyme structure and functional genomics.

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ABSTRACT:

Glutamine synthetase (GS) is the key enzyme involved in the assimilation of ammonia derived either from nitrate reduction, N_2 fixation, photorespiration or asparagine breakdown. A small gene family is encoding for different cytosolic (GS1) or plastidic (GS2) isoforms in legumes. We summarize here the recent advances carried out concerning the quaternary structure of GS, as well as the functional relationship existing between GS2 and processes such as nodulation, photorespiration and water stress, in this latter case by means of proline production. Functional genomic analysis using GS2-minus mutant reveals the key role of GS2 in the metabolic control of the plants and, more particularly, in carbon metabolism.

1. INTRODUCTION

1.1. Nitrogen Assimilation and Remobilization in Legumes

Nitrogen (N) is one of the most important nutrients for plants and, in natural soils, its availability is often a major limiting factor for plant growth. Of all the mineral nutrients, N is required in the largest quantities for the construction and maintenance of plant cells. Protein contains on average about 12% N by weight, and the whole dry matter of herbaceous plants typically contains from 1.5 to 4.5% N. This explains why 85–90 million tons of nitrogenous fertilizers are added to the soil worldwide annually [1]. Nitrogen is one of the most expensive nutrients to supply and commercial fertilizers represent the major cost in plant production. Furthermore, there is a serious concern regarding nitrogen loss in the field, giving rise to soil and water pollution. Incomplete capture and poor conversion of nitrogen fertilizer also causes global warming through emission of nitrogenous oxide. Lowering fertilizer input and breeding plants with better nitrogen use efficiency is one of the main goals of research on plant nutrition [2,3]. Thus, nitrogen availability is a particular challenge for plant survival, and different metabolic regulations and interactions have evolved to guarantee the strict economy of this essential nutrient during the plant life cycle.

The use of nitrogen by plants involves several steps, including uptake, assimilation, translocation and different forms of recycling and remobilization processes, all of them of crucial importance in terms of nitrogen utilization efficiency [2,3] (Figure 1). Primary nitrogen assimilation by plants involves the use of different forms of inorganic nitrogen (NO_3^- or NH_4^+), depending on nitrogen availability, plant species and adaptations. Alternatively, the symbiosis with bacteria enables also to several plant species, most notably legumes, to use atmospheric N_2, which is reduced to NH_4^+ in the nodules by the action of bacterial nitrogenase. In addition, efficient secondary ammonium assimilation must also exist in plants in order to reassimilate the ammonium ions that can be produced endogenously in the plants from processes such as photorespiration, phenylpropanoid biosynthesis, or amino acid catabolism. Photorespiration is probably the most important process in which high amounts of ammonium are released during the regeneration of 3-phosphoglycerate in the photorespiratory pathway [4]. In fact, the conversion of glycine to serine in the C2 cycle is probably the most important metabolic process that liberates ammonium in photosynthetic cells, at a rate that can exceed by 10-fold the rate of primary assimilation. Glutamine is the first organonitrogen compound that is synthesized in the plants as a result of both primary and secondary ammonium assimilation.

Depending on plant species, either glutamine or asparagine [5,6], is the preferred nitrogen compounds utilised to translocate the reduced nitrogen within the plant. Different legume species can use also ureides for N translocation. do Amarante *et al.* [7] have summarized very well our understanding as to when legumes use amides rather than ureides as nitrogen transport compounds in the xylem.

Among the different types of plant species, the Leguminoseae are second only to the Gramineae in importance to humans as a source of food, feed or livestock and raw materials for industry [8,9]. Legumes are the lynch pin of sustainable agriculture because they are able to fix nitrogen in a symbiotic association with *Rhizobium*, which provides these plants and subsequent crops with a free and renewable source of nitrogen (atmospheric N_2). It is estimated that between 40 and 60 million tons of N are fixed annually by cultivated legumes, which saves about US$ 10 billion in fertilizer [9]. Legumes account for approximately a third of the world's primary crop production, human dietary protein and processed vegetable oil [8,9]. Legumes are also able to establish beneficial symbioses with soil fungi that enable them to mine phosphorous and other essential nutrients from the soil more effectively. In spite of the importance of legumes in agriculture, increases in yield through breeding over the past few decades have lagged behind those of cereals. Numerous abiotic and biotic impediments continue to limit yield potential in legumes, including: drought, soil salinity, acidity and nutrient limitation; and various diseases and pests. Developing plants that are tolerant to these stresses remains an important aim of breeding programs [9]. While classical plant breeding can and will lead to further improvements in legume phenotypes in the future, the genomics revolution offers alternative and complementary approaches that can aid and accelerate plant breeding. Genomics and functional genomics, together with the more classical scientific disciplines of genetics, biochemistry, physiology, and molecular and cell biology, have already accelerated discoveries in legume molecular and systems biology. Unfortunately, agricultural legumes are relatively poor model systems for genetics and genomics research. Studies on most of the major leguminous crops are hampered by large genome sizes and other disadvantages (polyploidy, transformation or regeneration recalcitrancies, few or large seeds and seedlings, genome duplications, long generation times, *etc*.). As a result, two other species, *Lotus japonicus* and *Medicago truncatula*, have been adopted internationally as models for modern legume research [10–12].

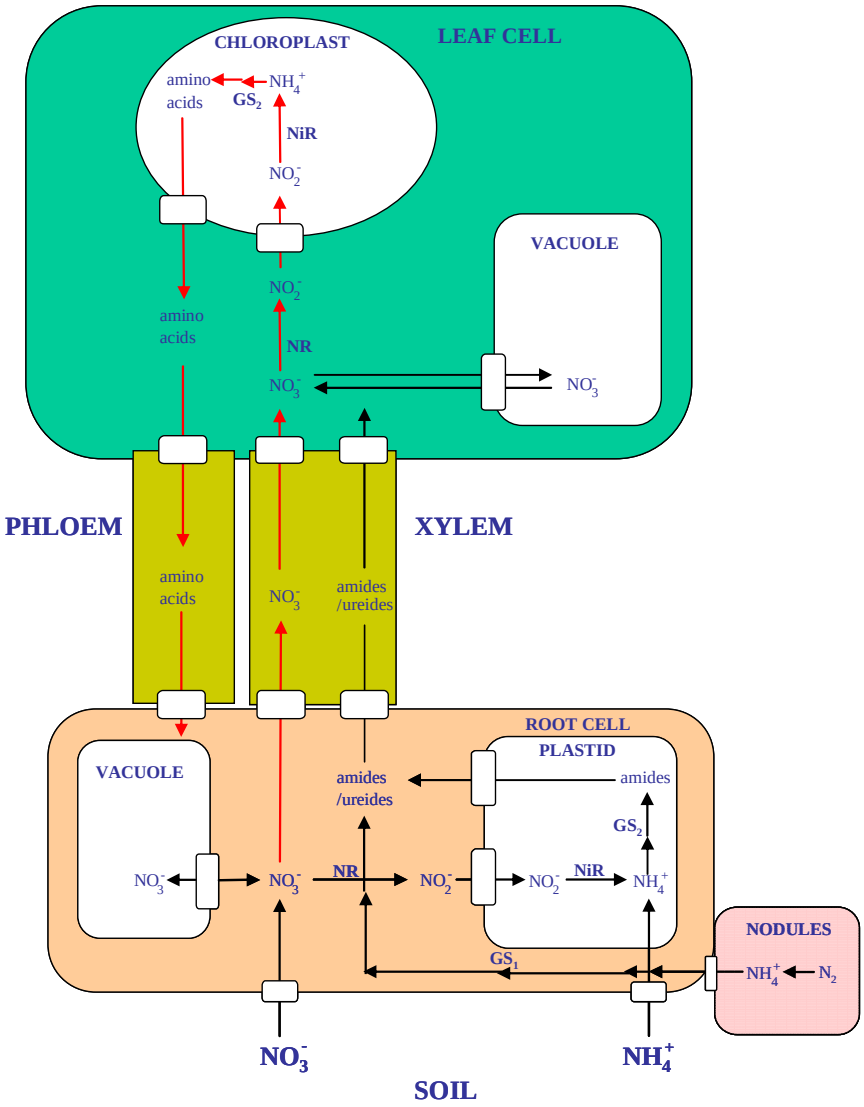

Figure 1. Nitrogen utilization in legume plants. Simple arrows represent single step reaction, while consecutive arrows represent multiple-step reactions. GS_1, cytosolic glutamine synthetase; GS_2, plastidic glutamine synthetase; NR, nitrate reductase; NiR, nitrite reductase.

Over the past 20 years, our research group has been working on nitrogen assimilation in the model legume *Lotus japonicus*. A previous review summarized the work carried out on the nitrate assimilatory process in this plant emphasizing the key importance of the root system in this regard [13,14]. In the present work we wish to present a global view of the most recent advances obtained by our group related to glutamine synthetase in this model plant and other legumes.

1.2. Glutamine Synthetase and Related Enzymes

Glutamine synthetase (GS, EC 6.3.1.2) is the key enzyme in charge of glutamine biosynthesis in nature. This enzyme catalyzes the incorporation into one molecule of glutamate of the ammonium derived either from primary (nitrate reduction, N_2 fixation) or secondary forms of nitrogen assimilation, at the expenses of ATP, according to the following reaction: L-glutamate + NH_4^+ + ATP → L-glutamine + ADP + P_i + H^+. Two types of GS isoenzymes do exist in plants: cytosolic (called GS1) or plastidic (called GS2). Different GS isoforms, either cytosolic or plastidic, have been reported, which have specific, apparently non-redundant, physiological roles in ammonium assimilation [15–20]. GS1 is localized in the vascular tissue and plays an important role in the assimilation of external ammonium, the ammonia derived from N_2 fixation and other sources of nitrogen, and in the remobilization of nitrogen during senescence [21,22]. Differential expression of a small multigene family (from 2 to 4 *GLN1* functional genes) is responsible for the behavior of the different cytosolic GS isoforms present in plants [20]. For example, three different cytosolic functional genes plus one pseudogene were found in *Phaseolus vulgaris*, where it was shown that one of this cytosolic genes (GSα) was majoritarily expressed in early developmental stages of the leaves, while a second one (GSβ) is expressed in leaves, roots and nodules, and a third gene (GSγ) is predominantly expressed in nodules coincidently with the onset of the nitrogen fixation process [15]. Conversely, plastidic GS2 is predominantly expressed in green tissues, and it has been demonstrated that this particular isoform has an essential role in the reassimilation of ammonium released by photorespiration [23,24], although its presence in non-photosynthetic tissues of temperate legumes have been also reported [25], as it is the case of *L. japonicus* [24] and *M. truncatula* [26]. Curiously, Taira *et al.* [27] also detected GS2 in *Arabidopsis* mitochondria. However, a single gene encoding for GS2 (*GLN2*) is mostly present in plants, as it happens in the case of the model legume *Lotus japonicus* [28,29], although a second gene encoding for GS2 was recently shown to be exclusively expressed in developing seeds from *Medicago truncatula* [30].

Other enzymes are in charge of the utilization of the glutamine synthesised by glutamine synthetase in order to achieve the synthesis of the rest of organonitrogen compounds required by plants. Glutamate synthase (GOGAT) is of crucial importance because it acts in tandem with GS for the synthesis of glutamate by means of the GS-GOGAT cycle. Two different types of GOGAT enzymes exist in plants called respectively Fd-GOGAT (EC 1.4.7.1) or NADH-GOGAT (EC 1.4.1.14), depending on the use of either ferredoxin or NADH as electron donors [31,32]. Of crucial importance too is asparagine synthetase (AS,

EC 6.3.5.4), which acts in conjunction with GS, GOGAT and aspartate aminotransferase (AAT, EC 2.6.1.1) for the synthesis of asparagine that can be used for N translocation in plants (Figure 2). In addition, we should also mention glutamate dehydrogenase (GDH, EC 1.4.1.2) as a complementary enzyme in charge of a reversible amination/deamination reaction which could lead to either the synthesis or the catabolism of glutamate. The role of GDH in glutamate metabolism in plants has been the subject of continued controversy. Except for particular stress conditions most reports indicate that GDH is mostly associated with the catabolic deamination of glutamate rather than a role in glutamate biosynthesis [32].

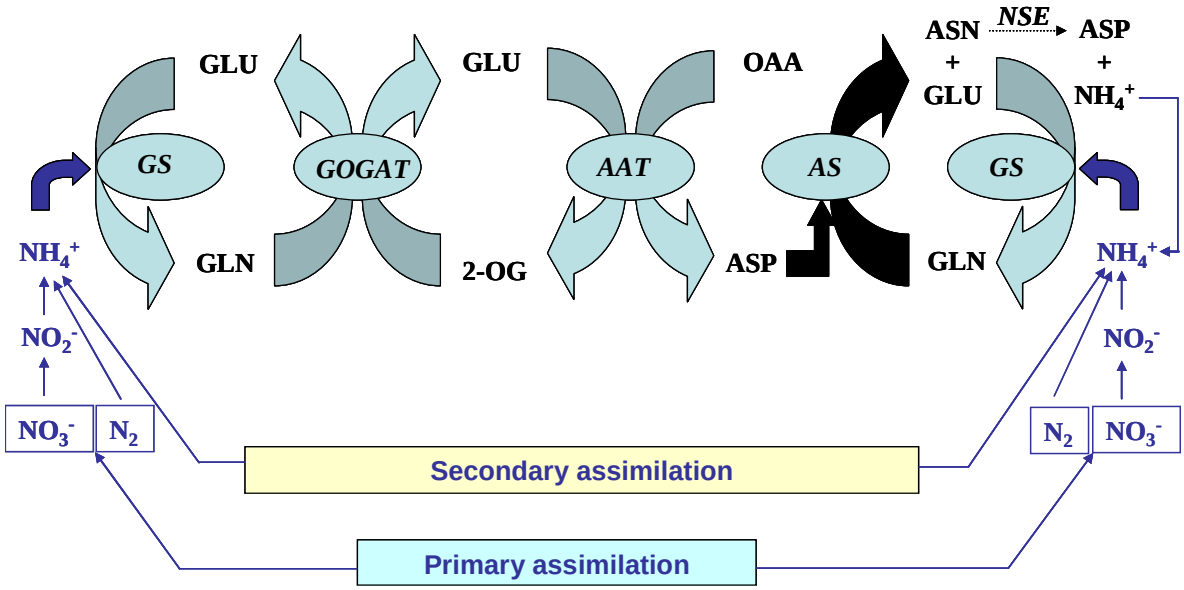

Figure 2. Enzymes involved in glutamine metabolism. GS, glutamine synthetase; GOGAT, glutamate synthase; AAT, aspartate aminotransferase; AS, asparagine synthetase; NSE, asparaginase.

2. Advances in Glutamine Synthetase Research

2.1. Enzyme Structure

GS is a ubiquitous enzyme found in all organisms through three different types of proteins: dodecameric GS-I (mostly found in prokaryotes); octameric or dodecameric GS-II (mostly located in eukaryotes), and hexameric GS-III (also found in prokaryotes). GS-I has a M_r of around 600,000 and is by far the best characterized of all GS types. The structure of GS-I from several organisms has been determined at atomic resolution [33–36] and it has been found to be a dodecamer built up by two back-to-back hexameric rings. The active site of GS-I, whose residues are conserved in all types of GS, is located between adjacent,

intra-ring monomers so that the oligomer possesses 12 active sites containing each one two metal ions (Mg^{2+} or Mn^{2+}) that are crucial to the enzymatic activity. Each monomer, with an average length of ~470 residues, is divided in two domains, each contributing to the active site of adjacent monomers. The smaller N-terminal domain contains mostly a sheet made by six antiparallel β-strands of which two form part of the active site. The larger *C*-terminal domain, which is mainly α-helical, contains six β-strands which hold most of the residues building the active site. The dodecamer is maintained mainly by hydrophobic interactions between the two hexameric rings.

The GS-II type, a smaller protein than GS-I (~370 residues average length), has been less studied than its prokaryotic counterpart, both in functional and structural terms. Nevertheless, the oligomeric state of GS-II has been the subject of study for more than two decades. Several models have been generated, based on electron microscopy and biochemical studies which point to GS-II as an octamer made by two superimposed rings formed by four identically placed subunits [37–41].

Three-dimensional reconstruction of recombinant homopolymeric α GS-II from *Phaseolus vulgaris* using electron microscopy and image processing, combined with other biochemical and biophysical data, revealed that this protein was an octamer built by two tetramers which are placed back-to-back and rotated 90° with respect each other [42]. The basic symmetry found for the three-dimensional structure (c2) and some biochemical data strongly suggested that the tetramers are formed by the interaction of two preformed dimers so that there are only two active sites per tetramer. The possible existence in this protein of four active sites and four ATP-binding regulatory sites was suggested [42]. Crystallization and X-ray structural determination of GS-II enzyme was achieved by first time for one particular isoform from maize (GS1a), which is remarkably stable [43]. The structure revealed a unique decameric structure and is formed by two back-to-back pentameric rings, with a total of 10 active sites, each formed between every two neighboring subunits within each ring, in a similar form to that described in bacteria, but containing three Mn^{2+} atoms in the middle of the catalytic cleft. A key residue responsible for the heat stability in this protein was found to be Ile-161. More recently, decameric eukaryotic GS II type enzymes from other biological sources have been also reported from humans or animals [44], yeast [45] or *Medicago truncatula* [46]. Remarkable differences between GS-II and GS-I type enzymes are notable in the mode of inter-ring subunit contact. The interface surface between the two pentameric rings of type II GS is much smaller than that of the hexameric rings of bacterial type I GS (Figure 3).

This is mainly due to the fact that the primary structure of type II GS has several internal deletions and a *C*-terminal truncation in comparison with the bacterial type I GS. Subunit interaction becomes very limited: only 4 hydrophobic interactions and 2 hydrogen bonds in type II GS1a from maize versus 37 hydrophobic interactions and 36 hydrogen bonds contributed by 43 residues in the case of type I GS [43].

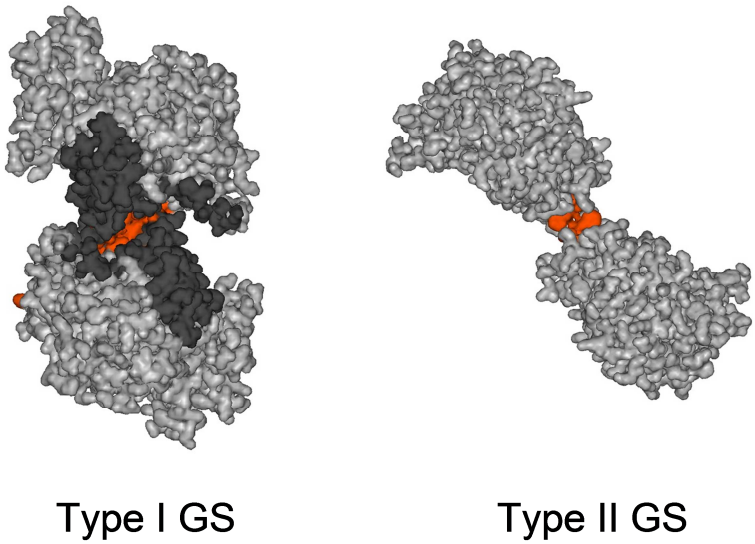

Figure 3. Differences in the amount of inter-ring subunit interactions of type I and type II GS. The figure shows in orange the main contact region among subunits existing in *Salmonella typhimurium* GS (type I GS) compared to GS1a from maize (type II GS) as drawn in a lateral view from their corresponding three dimensional structures.

It was determined in our laboratory that one particular cysteine residue (Cys159) of α-GS from *Phaseolus vulgaris* was essential for the enzyme structure and function [47]. Replacement of Cys159 by alanine or serine was sufficient to alter the subunit composition and quaternary structure of the GS enzyme molecule, resulting in a complete loss of enzymatic activity. In addition, treatment of this enzyme with sulfhydryl specific reagents such as pHMB (p-hydroxymercuribenzoate) or DTNB (5,5'-dithiobis-2-nitrobenzoate) confirmed that a strong inhibition of enzyme activity is produced, which is associated with a similar alteration of quaternary structure. Cys159 from *P. vulgaris* α-GS lies within the region equivalent to positions 146–186, that is critical for protein stability in the maize GS1a enzyme with a particular relevance of Ile161 [43]. However, amino acid replacement of Cys159 by alanine or serine in GS1a was not sufficient for the quaternary structure alteration of this protein (Estivill and Hase, personal communication). Other differences among both proteins were observed. For example, the effects of site-directed mutagenesis of Pro146 and Tyr150 on the quaternary structure and enzyme activity of α-GS from *P.*

vulgaris, were more intense than those of the corresponding homologue residues in GS1a from maize (Pro146 and Phe50) [48]. These residues in GS1a are mainly involved in the inter-ring subunit interactions [43]. All this suggests that particular differences must exist among the protein structures of different isoforms of higher plant GS, that may be responsible for the observed differences in the quaternary structures (octameric or decameric). While octameric structures may correspond to highly unstable proteins such as the recombinant homopolymeric α-GS from *P. vulgaris*, other decameric structures may correspond to highly stable forms of GS such as GS1a from maize.

Other site-direct mutagenesis studies on the α-GS from *P. vulgaris* were also carried out in our laboratory [49–51], combined with isothermal titration microcalorimetric and fluorescence studies [48,52]. A clear role for Asp56 and Glu297 in the reactivity towards the NH_4^+ in the catalytic mechanism of this protein was observed. It was also determined the essentiality of His249 and crucial importance of Arg316 in the interaction with ATP. Thus, the catalytic behavior of highly conserved amino acid residues from the active site of α-GS (type II GS) was found to be similar to that previously reported for type I bacterial GS [33–36]. Interestingly, different effects were produced either on biosynthetic or the transferase catalytic reactions of GS by many of the mutations examined. Therefore it can be concluded that there is a different role of active-site residues on the two catalytic activities commonly assayed by GS researchers. In addition, it was also demonstrated that site-directed mutagenesis of Asp56 residue, which is located far away from the ATP active site compartment, may also affect the ATP binding process. Moreover, different types of results indicate that the affinity for glutamate of the GS protein is highly susceptible to be altered. For example, mutations such as R316Q or D56E may result in important changes in the K_m for glutamate in α-GS from *P. vulgaris*. It was also demonstrated the crucial requirement of the metal cofactors in the maintenance of the quaternary protein structure and K_m for glutamate of plastidic GS from *L. japonicus* [53]. Other studies carried out in our laboratory have indicated that the K_m for glutamate of recombinant higher plant GS enzymes produced in *E. coli* may be substantially different from those of the same enzyme observed in higher plant crude extracts. This is the case of K_m for glutamate of recombinant plastidic GS2 from *L. japonicus*, which was found to be 6-fold higher than that from *L. japonicus* crude extracts [28]. All these results suggest the existence of different quaternary structure and/or conformational changes in higher plant GS, which may result either in a tense (low activity due to high K_m for glutamate) or relaxed (high activity due to low K_m for glutamate) conformation of GS. It is quite likely that different types of post-translational

modifications may be responsible for these changes. In fact, different mechanisms of post-translational modifications of plant GS have been reported, involving phosphorylation-dephosphorylation and 14-3-3 protein binding [54–58], thiol regulation [28] and, more recently, sumoylation [59] or tyrosine nitration [60]. The existence of GS post-transcriptional and/or post-translational regulatory events is also suggested by other types of work using transgenic plants [61–64]. Our recent work in collaboration with the group of Dr. Georgina Hernández (ICG, Cuernavaca, México) [48] shows also that very high levels of GS protein expression may not correspond with high levels of enzyme activity in transgenic plants from *L. japonicus*. However, there is not yet a clear idea on how the different post-transcriptional or post-translational regulatory mechanisms may affect the enzyme activity of GS in plants.

2.2. Use of Mutants for the Study of Plastidic GS Functionality

The role of the plastidic GS isoform in plants has been historically defined as the reassimilation of the ammonium produced by the photorespiratory cycle [23,24,65,66]. In fact, mutants lacking plastidic GS are conditionally lethal, in the sense that they grow normally under a CO_2-enriched atmosphere (>0.2%) where photorespiration is suppressed but show serious stress symptoms when transferred to normal air, an atmosphere that permits photorespiration (eventually leading to death if the plants are grown in these conditions for a long time). Photorespiratory mutants were first discovered by Sommerville and Ogren [67]. Different types of photorespiratory mutants were later on isolated that can be impaired in different steps of the C2 photorespiratory cycle. Interestingly, while mutants of some enzymes of the cycle like Fd-GOGAT were isolated in different plant species like *Arabidopsis*, barley, pea and tobacco [65,68–70], mutants of plastid GS were only obtained in barley [23] and *Lotus japonicus* [24], making the latter ones the only GS2 mutants in a legume described so far.

Two different *L. japonicus* plastid GS mutants, initially called *Ljpr1* and *Ljpr2*, were isolated in our laboratory by Orea *et al.* [24] after screening approximately 30,000 plants treated with the mutagen ethyl-methanesulphonate (EMS). Both mutants were allelic and showed a mendelian inheritance of a single recessive trait. The mutants accumulated high levels of ammonium when grown under photorespiratory-permitting conditions and had normal levels of the cytosolic GS isoform. However, chlorosis and necrosis of the edges of the leaves appeared after several days of transfer from high CO_2 to normal air atmosphere (air-sensitivity phenotype). Mutant plants could be rescued if transferred back to a CO_2-enriched atmosphere, but long-term exposure to normal air eventually caused the falling of the leaves, beginning from the younger ones. The main difference between the two mutants was that in *Ljpr1* the GS2 polypeptide was

present, though in a lower amount with respect to the WT plants, while in *Ljpr2* no GS2 polypeptide was detected. Since both mutants had normal levels of GS1 protein and activity, it was clear that this isoform is not able to compensate for the lack of the plastidic one. Moreover, the similar levels of total GS activity in the leaves of *Ljpr1*and *Ljpr2* (about one third than the WT), together with the similar air-sensitivity of the two mutants, strongly suggested that the GS2 polypeptide detected in the *Ljpr1* mutant lacked enzyme activity. In order to test this hypothesis, and to gain further insight into the functionality of plastidic GS in *L. japonicus*, a molecular analysis of the two mutants was later on carried out [28]. The transcription of the plastidic GS gene was normal in both mutants, indicating that the mutants were affected at the post-transcriptional level. Further sequencing of WT and mutant *GLN2* cDNAs revealed that each of *Ljpr1* and *Ljpr2* carried a different point mutation in one exon of the GLN2 gene encoding for plastidic GS (leading to G85R and L278H amino acid replacements respectively). Since both mutant plants were affected at the level of the *LjGLN2* gene sequence, the mutants were called *Ljgln2-1* and *Ljgln2-2* according to the standard nomenclature [28]. Using different types of approaches, it was demonstrated that the plastidic GS polypeptide from both mutants lacked any detectable enzyme activity. Analysis of the purified mutant enzymes recombinantly produced in *E. coli* indicated that the GLN2-1 mutant polypeptide was assembled into a functional, though unstable GS oligomer. In contrast, the GLN2-2 polypeptide was not able to acquire a proper quaternary structure and was rapidly degraded, in agreement with the different GS2 polypeptide levels observed in extract from leaves of the mutants.

Homozygous *Ljgln2-1 or Ljgln2-2* mutant plants resulting from the progeny of two consecutive back-crosses of each of the mutants with the wild-type were produced. These mutant lines, specifically lacking of plastidic GS, were further utilized to analyze different aspects of plastid GS functionality in *L. japonicus* plants as follows.

2.2.1. Plastidic GS and Photorespiration Transcriptomics

In addition to its obligatory relationship with photosynthesis, the photorespiratory cycle interacts with several other primary and secondary pathways in the cell, including the Calvin cycle [71], nitrogen assimilation, respiration and one carbon metabolism [72] as well as redox signaling [73]. Given this intertwining of photorespiration with several other routes, it was interesting to study the effect of the impairment of the photorespiratory cycle on the overall cellular metabolism. For this reason, we made use of the *Ljgln2-2* mutant in order to determine if the effect of a transfer from suppressed to active

photorespiratory conditions could affect the transcriptome of *L. japonicus*. The *Ljgln2-2* mutant was chosen since it lacked of any detectable levels both of plastidic GS activity and polypeptide. A preliminary study was carried out in leaves of WT and mutant plants that were grown for 45 days in CO_2-enriched atmosphere and then transferred to normal air conditions. The transcriptomic analysis was carried under control conditions (CO_2-enriched atmosphere) and after 2 days of exposition to normal air. Transcriptomic data were obtained using the recently developed Affymetrix GeneChip® Lotus1a520343, that contains 52,749 unique probesets. A probeset is an oligonucleotide designed to measure the expression of a known or predicted sequence of mRNA. Several probesets may correspond to a same gene in such a way that most of *L. japonicus* gene transcripts are analyzed in a single DNA chip. Changes in gene expression between WT and *Ljgln2-2* plants were analyzed by a significance-based comparison applying a false discovery rate (FRD) < 0.05, using three different biological replicates.

The total number of probesets modulated by the 2-days shift to photorespiratory conditions was much higher for the mutant plants: 5,785 probesets were changed exclusively in the *Ljgln2-2* genotype compared to the 655 that changed exclusively in the WT (Table 1). In both genotypes, the number of down-regulated probesets was higher that the number of up-regulated ones. The higher number of probesets modulated for the mutant plants indicates that impairment of the photorespiratory cycle has a vast effect on leaf metabolism. On the other hand, 825 modulated probesets were common to both genotypes (Table 1). These probesets probably represents the core/common response of *Lotus japonicus* to photorespiration that is not dependent on the presence of plastidic GS.

	WT only	**Shared**	***Ljgln2*-2 Only**
Total Probesets	655	825	5785
Up-Regulated	155	384	2470
Down-Regulated	500	441	3315

Table 1. Probesets modulated by 2-days transfer from CO_2-enriched (suppressed photorespiration) to normal air (active photorespiration) atmosphere in the leaf transcriptomics of either WT or *Ljgln2-2* mutant plants.

A preliminary analysis of this dataset was carried out focusing on the ten more up-regulated and down-regulated genes (Table 2). The fold-change values for the top up- and down-regulated genes in the mutant were higher than the corresponding values for the WT plants. This probably reflects the higher levels of cellular stress present in this genotype under active photorespiration.

Two groups of genes were highly represented amongst the most induced in both genotypes: genes involved in flavonoid biosynthesis and in redox metabolism (Table 2). Three genes involved in flavonoid biosynthesis were highly induced in the WT (polyketide reductase, isoflavone reductase and 2-hydroxyisoflavanone synthase, corresponding to probesets chr2.CM0191.49.2, chr2.CM0249.88 and TM0802.13 respectively). Genes for flavonoid biosynthesis were also induced in the mutant, three of them encoding for chalcone synthase (probesets chr3.CM0590.56, chr2.CM0018.54 and Ljwgs_099009.1) in addition to the gene encoding for 2-hydroxyisoflavanone synthase that was induced also in the WT. Flavonoids are a vast class of secondary metabolites involved in an array of processes, including plant–pathogen interactions, pollination, light screening, seed development and allellopathy. Moreover, most flavonoids show an important anti-oxidant capacity [74]. Many of the genes involved in flavonoid biosynthesis are induced under biotic or abiotic stress. This is probably aimed to increase the production of flavonoids in order to scavenge the increased amount of reactive oxygen species generated under these conditions. The induction of flavonoids biosynthesis may then suggest that the transfer of both plant genotypes to active photorespiratory conditions may be associated to increased levels of oxidative stress. According to this idea, several genes related to redox metabolism were highly induced in both genotypes. A gene encoding for glutathione-S-transferase and one for alpha-dioxygenase were amongst the ten most induced both for WT and *Ljgln2-2*. Interestingly, the alpha-dioxygenase gene induced in both genotypes (probeset Ljwgs_903636.1) was similar to the alpha-DOX1 fatty acid dioxygenase from *Arabidopsis* (transcribed unit: At3g01420), that is involved in protection against oxidative stress and cell death [75].

WT			
Probeset	**log_2 FC**	**Description**	**Similar to**
Up-Regulated			
chr2.TM0641.8	1.98	NAC domain protein	AT1G69490
chr5.CM0341.27	1.96	AP2-EREBP Transcription factor	AT3G23240
chr2.CM0191.49.2	1.91	Polyketide reductase	AT1G59960
Ljwgs_093636.1	1.87	Alpha-dioxygenase	AT3G01420
Ljwgs_050995.1	1.77	Methyltransferase protein	AT3G11480
Ljwgs_080010.1	1.73	Pleiotropic drug resistance protein	AT1G15520
chr4.CM0256.39	1.73	Cytochrome P450	AT4G37370
chr2.CM0249.88	1.72	Isoflavone reductase	AT4G39230
TM0802.13	1.69	2-Hydroxyisoflavanone synthase	AT5G06900
Ljwgs_018999.1	1.64	Glutathione S-transferase	AT2G29420
Down-regulated			
Ljwgs_108871.1	−2.74	Hypothetical protein	AT3G02550
Ljwgs_035693.2	−2.46	Hypothetical protein	AT3G20810
Ljwgs_089359.1.1	−2.02	Early flowering 4 protein	AT2G40080
chr3.CM0711.3.2	−1.93	Unknown protein	AT4G10270
chr2.CM0191.60	−1.83	Nlj21	-
Ljwgs_080939.1	−1.81	Beta-glucosidase like protein	AT2G44480
chr3.TM0426.3	−1.75	Hypothetical protein	AT5G22580
chr1.CM0398.23.1	−1.66	Gibberellin induced protein	AT1G74670
chr3.CM0155.27	−1.64	Peroxidase	AT1G05260
TC17223	−1.64	Hypothetical protein	-

Table 2. Top 10 genes up- or down-regulated by active photorespiration in WT and *Ljgln2-2*.

WT			
Ljgln2-2			
Probeset	**log_2 FC**	**Description**	**Similar to**
Up-Regulated			
Ljwgs_044797.1	5.37	60S ribosomal protein	AT1G26910
chr3.CM0590.56	4.52	Chalcone synthase	AT5G13930
Ljwgs_036303.1	4.43	NAC domain protein	AT4G27410
TM0802.13	4.35	2-Hydroxyisoflavanone synthase	AT5G06900
chr2.CM0250.2	4.34	MYB transcription factor	AT4G37260
gi45637799	4.26	Hypothetical protein	-
chr2.CM0018.54	4.19	Chalcone synthase	AT5G13930
Ljwgs_099009.1	4.06	Chalcone synthase	AT5G13930
Ljwgs_093636.1	4.04	Alpha-dioxygenase	AT3G01420
chr5.CM0909.51	4.03	Glutathione S-transferase	AT2G29420
Down-regulated			
chr1.CM0001.63	−4.66	Probable 2-Isopropylmalate synthase	AT1G74040
Ljwgs_091497.1	−2.97	Myo-inositol-1-phosphate synthase	AT5G10170
TM0810.14	−2.88	Cytochrome P450	AT2G45550
chr1.CM0398.23.1	−2.80	Gibberellin regulated protein	AT1G74670
TM1614.14.1	−2.60	Hypothetical protein	AT1G59960
Ljwgs_028558.1	−2.55	Pectate lyase	AT4G24780
Ljwgs_062989.1	−2.54	Terpene synthase	AT4G16730
chr3.CM0142.55	−2.52	Hypothetical protein	AT5G20190
chr1.CM0001.70.2	−2.45	Hypothetical protein	AT5G13750
Ljwgs_043433.1	−2.35	Benzoyl transferase	AT5G17540

Table 2. *Cont.*The table shows the probesets corresponding to the 10 genes that showed higher extent of modulation (FDR < 0.05) in their transcript levels in the leaves of *L. japonicus* plants after the transfer for 2 days from 0.7% (v/v) CO_2 (suppressed photorespiration) to normal air (active photorespiration) conditions as determined by transcriptome analysis using Affymetrix genechips. The log_2 of the fold-change is indicated together with the description of the most probable match and the most similar Arabidopsis gene. The following databases were used for the search: the Kazusa DNA research institute (Available online: http://www.kazusa.or.jp/lotus/blast.html; accessed on March 16, 2012), Non-redundant protein sequences (Available online: http://blast.ncbi.nlm.nih.gov/; accessed on March 16, 2012), TAIR (Available online: www.arabidopsis.org; accessed on March 16, 2012) and the Legume transcription factor database (Available online: http://legumetfdb.psc.riken.jp/index.pl; accessed on March 16, 2012).

Besides of the recycling of the glycolate-2-phosphate produced by the oxygenation reaction of the RUBISCO, the photorespiratory cycle also plays an important role in stress protection since it consumes ATP and reducing equivalent, preventing the over reduction of the photosynthetic electron transport which may result in the formation of reactive oxygen species [71]. It is then not surprising that the transfer of *L. japonicus* plants from photorespiratory-suppressed to photorespiratory-active conditions causes a response of flavonoid and redox metabolism. However, this response was not limited to the mutant with an impaired photorespiratory cycle but was also extended to the WT plants. This may indicate that, even in plants with normal levels of photorespiratory enzymes, the flux towards the photorespiratory pathway is associated with some levels of oxidative stress. Alternatively, it may also be possible that WT plants that have been grown under CO_2-enriched atmosphere for more than one month may have lower-than-normal levels of some photorespiratory enzymes.

The two most up-regulated genes in the WT plants corresponded to transcription factors (TFs): a NAM, ATAF1/2 and CUC2 (NAC) domain TF (probeset chr2.TM0641.8) and an APETALA2 (AP2) and ethylene-responsive element binding proteins (EREBPs) family one (probeset chr5.CM0341.27). Two TFs were also induced in the mutant, a distinct domain one (probeset Ljwgs_036303.1) and one belonging to the myeloblastosis (Myb) family (probeset chr2.CM0250.2). The modulation of these transcription factors may be triggered either by the reactivation of photorespiration or by the diminishment of carbon availability in both genotypes. In the case of the mutant, changes could be expected also by the metabolic consequences of the impaired photorespiratory route. The fact that different TFs were modulated in WT and mutant plants suggests a role for plastidic GS in the regulation of photorespiratory metabolism. Many repressed genes encoded for hypothetical proteins of unknown function. Future experiments should be aimed to the characterization of these genes and their corresponding gene products.

In summary, the transcriptomic study carried out reveals that the transfer of *L. japonicus* plants from photorespiratory-suppressed to photorespiratory-active conditions results in important changes in gene expression. Some transcription factors, mainly of the Myb and NAC-domain families, were among the most affected transcripts. Other stress-related pathways associated to oxidative stress and flavonoid metabolism were also particularly modulated. This response was greatly enhanced as a consequence of deficiency in plastidic GS, thus indicating the crucial significance of plastid GS in photorespiratory metabolism transcriptomics.

2.2.2. Plastidic GS and Nitrogen Nutrition

Plants can use various forms of combined nitrogen, most importantly the ions nitrate and ammonium (Figure 1). Despite of the fact that more energy is needed for the assimilation of nitrate, most plants prefer NO_3^- over NH_4^+. With the exception of ammonium tolerant species, the availability of NH_4^+ alone as nitrogen source, as well as the internal production of NH_4^+ by processes like photorespiration [76] may result toxic to the plant. Notably, the toxic effect of external ammonium can be partially relieved by co-provision of nitrate, the so-called mixed nitrate plus ammonium nutrition.

A fascinating and still poorly understood aspect of nitrogen nutrition is that in most cases the growth of a plant on NH_4NO_3 can surpass the maximal growth compared to either NO_3^- or NH_4^+ alone. This relief of NH_4^+ toxicity by NO_3^- may be related to a synergism between the signaling routes of NH_4^+ and NO_3^- [77]. Moreover, several genes are modulated exclusively when the nitrogen source is NH_4^+, NO_3^- or NH_4NO_3 [78].

As reported in this paper, the absence of plastidic GS in the *L. japonicus* mutant *Ljgln2-2* is associated with important transcriptomic changes under particular conditions like active photorespiration. We have examined if plastidic GS may also play a role in the transcriptional response of *L. japonicus* to the availability of different nitrogen sources. For this purpose, a study of the expression levels of key genes for nitrogen metabolism was carried out in leaves of WT and *Ljgln2-2* mutant plants that had been grown for 35 days in CO_2-enriched atmosphere and different nitrogen sources (8 mM KNO_3, 8 mM NH_4Cl or standard Hornum mixed nutrition, which consisted of 5 mM KNO_3 plus 3 mM NH_4Cl). The expression of genes coding for glutamine synthetase, glutamate synthase, asparagine synthetase and glutamate dehydrogenase was monitored by qRT-PCR.

Gene-specific oligonucleotides were synthesized based on sequences found in the available databases.

The comparison of the expression levels of the genes analyzed between WT and mutant plants is presented in Table 3 using a color code: boxes in green indicate higher expression in the WT than in the mutant while boxes in purple indicate higher expression in *Ljgln2-2*. Three different sequences corresponding to cytosolic GS were analyzed. Two of them (*LjGS1.1* and *LjGS1.3*) were more expressed in the mutant when the nitrogen source was either nitrate or ammonium. On the other hand, *LjGS1.3* was more expressed in the WT under

mixed nutrition. *LjGS1.2* had a very different behavior with respect to the other two cytosolic GS genes as it was more expressed in the mutant but only under mixed nutrition. The two genes coding for plastidic GS and Fd-GOGAT (*LjGLN2* and *LjGLU1*) were more expressed in the mutant with nitrate or ammonium, but their transcription was higher in the WT under mixed nutrition. This result suggests that *LjGLN2* and *LjGLU1* are regulated in a common fashion, as confirmed by studies in other plants [79]. In the case of the two sequences found coding for NADH-GOGAT (*LjGLT1* and *LjGLT2*), the expression of both genes was higher in *Ljgln2-2* under mixed nutrition. Interestingly, the regulation of the two genes coding for asparagine synthetase in *L. japonicus* (*LjAS1* and *LjAS2*) was the same as *LjGLN2* and *LjGLU1*. Since plastidic GS and Fd-GOGAT are the enzymes that carry out the reassimilation of photorespiratory ammonium in the leaves, this parallelism may suggest a link between asparagine biosynthesis and the photorespiratory cycle. Evidence of an involvement of asparagine in photorespiration also comes from early studies using metabolite labeling [80]. Finally, four sequences corresponding to GDH were found in *L. japonicus* DNA sequences databases.

The expression of *LjGDH3* was higher in the mutant plants under all the different nitrogen nutrition considered, while *LjGDH4* showed higher expression in the mutant only under mixed nutrition. On the other hand, the expression of *LjGDH1* and *LjGDH2* was undetectable in leaves (not shown). It is interesting to notice that for most genes the transcriptional effect of the deficiency in plastidic GS was very similar in the case of plants grown under NO_3^- and NH_4^+ but different, and in several cases opposite to the effect produced in NH_4NO_3-grown plants. This was the case of the *LjGS1.3*, *LjGLN2*, *LjGLU1*, *LjAS1* and *LjAS2* genes. These results suggest a role for plastidic GS in the distinctive response to mixed nutrition of the nitrogen assimilatory genes in *L. japonicus*, and may imply a role of plastidic GS in the signaling events related to the presence of different nitrogen sources.

Name	**Probeset**	**Nitrogen source**		
		NO_3^-	**NH_4^+**	**NH_4NO_3**
LjGS1.1	TM0053.11	2.71	2.69	0.93
LjGS1.2	gi1246767	0.95	0.84	1.88
LjGS1.3	Ljwgs_019428.1	6.22	3.94	0.22
LjGLN2	gi18266052	8.99	4.96	0.19
LjGLU1	chr1.CM0009.24	4.77	2.64	0.25
LjGLT1	Ljwgs_035611.1	0.71	1.40	1.36
LjGLT2	Ljwgs_037992.1	1.06	1.70	1.23

LjAS1	gi897770	4.91	2.58	0.30
LjAS2	gi897772	4.57	3.64	0.73
LjGDH3	Ljwgs_035272.1	2.32	1.73	1.46
LjGDH4	Ljwgs_009442.1	1.09	1.04	1.13

Table 3. Changes in the transcript levels for different isoforms of GS, GOGAT, AS and GDH in leaves of WT and *Ljgln2-2* mutant plants grown in the presence of different nitrogen sources. Transcript levels were determined by qRT-PCR using three independent biological replicates. Data are reported as the ratio of transcript levels between *Ljgln2-2* and WT plants, previously standardized to housekeeping genes. Genes that are more expressed in the mutant than in the WT, according to Student's t test ($p < 0.05$), are highlighted in purple, while boxes in green indicate significantly higher expression in the WT.

2.2.3. Plastidic GS, Photorespiration and Nodulation

How photorespiratory metabolism affects nodulation has not been sufficiently studied. A recent study gives new insights into the influence of photorespiratory metabolism and GS2 on nodule function using photorespiratory mutants lacking GS2 isoenzyme [81]. It is generally assumed that GS1 is the GS isoenzyme responsible for primary assimilation of the NH_4^+ released by bacteroids [15]. Different approaches have been utilized to study the physiological impact of altered GS activity in the nodules, using transgenic plants showing a reduction or overexpression of GS1 enzyme activity [82,83]. The presence of plastid GS2 isoform in nodules has been reported for *Medicago truncatula* [84] and *L. japonicus* [81]. The studies carried out with GS2 mutants from *L. japonicus* determined that GS2 accounts for up to 40% of total nodule GS activity [81], a relatively unexpected result, since no decrease was observed in these mutant plants with regard to total GS activity from roots [24], a closely related organ to nodules. A comparative Western blot analysis of WT and GS2 mutant nodules showed a similar level of the GS1 polypeptide in WT, *Ljgln2-1* and *Ljgln2-2* genotypes, whereas the GS2 polypeptide was present in nodules of WT plants and was undetectable or only a faint band could be observed in both mutant genotypes. This indicated that the mutants were not only deficient in the GS2 isoform from leaves and roots as previously reported, but also in the GS2 isoform from nodules, which must be responsible for at least 40% of GS activity in this organ. So GS2 has a very significant contribution to the total levels of GS activity present in nodules of *L. japonicus* plants.

The fact that mutant plants lacking GS2 were still able to establish symbiosis and fix nitrogen in a similar way than WT indicated that the GS2 isoenzyme was not essential for nodulation and nitrogen-fixation processes under

non-photorespiratory conditions. Therefore, it was confirmed that GS1 is sufficient for an efficient assimilation of ammonium derived from N_2 fixation. Nevertheless, the influence of photorespiratory metabolism on nodulation could be also determined in WT and GS2 mutant plants, which were grown in CO_2-enriched atmosphere (photorespiration-suppressed conditions) and then transferred to low CO_2 atmosphere (photorespiration-active conditions) [81]. This transfer substantially affected the number of nodules obtained, the FW of these nodules, and the levels of ARA (acetylene reduction activity), which were highly reduced compared with the plants maintained under CO_2 enrichment. At early developmental stages, mutant plants were affected in a very similar way to WT, thus indicating that the additional lack of GS2 did not have a significant influence on nodulation at this developmental stage. However, the transfer of the plants from high CO_2 to air atmosphere at a later stage of plant development resulted in a dramatic reduction in nodule FW (40 to 60% decrease) and ARA activity (60 to 85% decrease) The mutant nodules were considerably more affected than the WT ones, indicating that GS2 deficiency affected nodule mass and function, particularly under photorespiratory active conditions. It was therefore demonstrated that the photorespiratory activity of the plant generates a negative influence in nodule formation, development, and function, at later stages of growth, particularly in GS2 deficient plants [81].

Sucrose is the predominant sugar detected in nodules of *L. japonicus* WT and GS2 mutant plants [81,85]. This carbohydrate is the first photosynthate supplied to nodules and, consequently, nitrogen fixation in legume nodules is highly dependent on the supply of sucrose delivered from the phloem [86]. When carbon levels are high, starch is the main stored compound. In fact, electron micrographs have previously suggested the presence of high starch content in nodules grown at elevated CO_2 [87]. It has been reported that nodulated plants growing under atmospheric CO_2 enrichment showed enhanced whole-plant growth and increased nodule biomass, and the nodules showed higher sugar and starch contents as well as enhancement of some activities related to nodule carbon metabolism and increased ARA [88–90]. These results indicated that under CO_2 enrichment a high amount of carbon must be fixed and directed to nodules. In other studies, alfalfa plants with CO_2 application tended to form fewer and bigger nodules, but in this case, the CO_2 was applied around the root and nodule compartment [91] and not around the shoots, as in other studies. However, neither a short- nor a long term-effect on nodule ARA-specific activity has been observed in these plants [90,92]. By contrast, it has recently been described that ARA-specific activity increases in nodules of plants subjected to high CO_2 treatment applied only to the root and nodule compartment [91].

In *L. japonicus* plants, carbohydrate analysis revealed a decrease of the levels of glucose, fructose, sucrose and starch in plant nodule extracts when plants growing in CO_2-enriched atmosphere were transferred to low CO_2 conditions [81]. These studies revealed that when photorespiration begins, the CO_2 assimilation diminishes, as it is also the available photosynthate content that can be directed to nodules. The transfer to air conditions lead to alterations of carbon metabolism and therefore bacteroids were limited in carbon compound levels causing reduction of atmospheric nitrogen fixation, and thus indicating that the photorespiratory activity of the plant influences negatively the nitrogen fixation rate by limiting the carbon availability. The decrease of carbohydrate level, particularly starch and sucrose, in plant nodule extracts was more remarkable in nodules from mutant plants transferred to air (where a reduction of about 65 to 80% was observed) than in nodules from plants grown under CO_2-enriched atmosphere. Interestingly, under photorespiration-suppressed conditions, the relative levels of sucrose were considerably lower (around 60% reduction) in nodules from mutant plants than in those from WT. These results revealed the existence of alterations in carbon metabolism of mutant nodules, under both photorespiratory- active and -suppressed conditions.

Recent reports established that antisense inhibition of NADH-GOGAT activity impairs carbon and nitrogen metabolism in alfalfa nodules [93]. The requirement of carbon skeletons for ammonium condensation and the supply of reducing equivalents as products of photosynthesis, respiration, and photorespiration pathways are well known [3,94]. It was also demonstrated that the general nitrogen nutritional status of *L. japonicus* plants can strongly affect the competence for nodule formation [95]. The reduction in number of nodules observed in the mutant plants after the shift to air conditions could be explained by the increased amount of non-assimilated ammonium representing an inhibitory signal for the nodule formation mechanism [95,96]. Studies with photorespiratory mutants containing normal levels of GS1 but specifically lacking of GS2 activity showed that the lack of GS2 substantially increased the negative effect of photorespiration on the nodulation process and nitrogen fixation in mutant plants. In addition, optical microscopy data revealed alterations in mutant nodules such as development restrictions, disappearance of starch granules in the non-infected cells, increase in size of vacuoles and appearance of regions with lower bacteroid density in the cytoplasm of infected cells, indicative of a cell lysis process [81].

In conclusion, the *Lotus-Rhizobium* symbiotic process was highly affected under active photorespiratory conditions. The studies with GS2 mutants deficient

in photorespiratory ammonium assimilation emphasized how a “nitrogen” assimilation defect affected “carbon” metabolism and nodule function in *L. japonicus*. Interestingly, it was observed a clear-cut deficiency in carbon metabolism in mutant plants maintained under CO_2-enriched atmosphere at later developmental stages, with a strong reduction in the sucrose nodule content as well as a significant decrease in starch, glucose and fructose levels compared with WT plants. Thus a role of GS2 in the C/N balance of *L. japonicus* plants, independent of the photorespiratory activity of the plant, can be established [81].

2.2.4. Plastidic GS and Drought Stress Transcriptomics

Osmotic stress associated with salinity and drought is one of the most serious problems that reduces crop productivity world-wide. Legumes represent about one third of the world’s primary crop production and are a valuable protein source for both human and animal feeding. In particular, several species of the genus *Lotus* are used as pasture in temperate regions, where the plants can be exposed to sudden periods of drought. For this reason, the search for genes that may contribute to stress tolerance in *Lotus* is of particular significance. Such kinds of studies have been carried out in other plants using DNA chips, which permit the quantification of the transcription of thousand of genes. A great number of genes that are modulated by drought have been identified, particularly in the model plant *Arabidopsis thaliana*, but the function of only a limited number of them is known [97]. However, the recent availability of the Affymetrix Lotus1a520343 Genechip® permitted to aboard the study of the transcriptomic response to drought also in this model legume. It was of particular interest to determine the drought stress transcriptome not only in WT plants but also in the *Ljgln2*-2 mutant. In fact, it has been described in other plants that GS may play an important role in the response to abiotic stress. For example, a role for cytosolic GS was clearly established in proline production [98]. On the other hand, overexpression of the plastidic GS isoform in non-legume plants like tobacco and rice resulted in enhanced tolerance to photooxidation [66] and to salt stress [99].

In order to study the role of *L. japonicus* plastidic GS in the response to drought stress, WT and *Ljgln2-2* mutant plants were submitted to 4 days of water deprivation. The *Ljgln2-2* mutant was chosen since it lacked of any detectable levels both of plastidic GS activity and polypeptide. WT and mutant plants were grown under a CO_2-enriched atmosphere in order to avoid the concomitant effect of drought and photorespiration on the mutant. The lack of plastidic GS had several important effects in the response of the *Ljgln2-2* mutant to water

deprivation. Mutant plants with the same level of hydric deficit as the WT showed higher thiobarbituric acid-reactive species (TBARS) content, indicating a higher level of oxidative membrane damage in *Ljgln2-2* under drought [100]. Moreover, the metabolism of proline, one of the most common compounds produced by plant cells in response to abiotic stress [101], was deeply altered in the mutant. In fact, *Ljgln2-2* plants accumulated less proline when compared to the WT ones at a similar hydric deficit levels and significant differences in the expression of the genes for proline metabolism were observed. These data established for the first time a link between plastidic GS and proline metabolism [100].

To gain further insight into the role of plastidic GS in the response to drought stress, the transcriptomes from leaves of WT and mutant plants after 4 days of water deprivation were compared with the transcriptomes of well-watered plants in order to identify the genes modulated by drought. Of the 52,749 probesets contained in the *Lotus japonicus* Genechip®, 538 corresponded to genes modulated by drought exclusively in the WT plants and 5,845 exclusively in the mutant, while 2,070 probesets were changed in both genotypes (Table 4).

	WT Only	**Shared**	***Ljgln2*-2 Only**
Total Probesets	538	2070	5845
Up-Regulated	207	946	3636
Down-Regulated	331	1124	2209

Table 4. Probesets elicited by 4 days of water deprivation in the leaf transcriptomics for either WT or *Ljgln2-2* mutant plants.

A global analysis of the *Lotus* drought transcriptomic dataset was presented in a previous work [100]. Here, we will just focus on the top 10 genes that were most up-regulated and down-regulated in either WT or *Ljgln2-2* genotypes (Table 5). The sequences corresponding to the most highly modulated genes were used to search for similar ones in several databases in order to determine the function of each gene product. The genes identified in this way were related to different metabolic processes. For example, different genes involved in hormone metabolism were up-regulated: 1-aminocyclopropane-1-carboxylate (ACC) synthase (probeset chr1.TM1635.18), the enzyme that catalyzes the rate-limiting step in ethylene biosynthesis, was induced in both genotypes, suggesting increased ethylene biosynthesis under drought stress. The function of ethylene during drought is however a matter of controversy: ethylene alone seems to promote stomatal closure, a condition that reduces water loss, but

it has also an inhibitory effect on the abscisic acid-stimulated stomatal closure suggestive of a yet little-understood crosstalk between different hormones during the stress response [102]. On the other hand, 12-oxyphytodienoate reductase, an enzyme involved in jasmonic acid biosynthesis, was induced only in the mutant (probeset TM0763.11).

Two different genes involved in the production of molecules with protective function were the most up-regulated in the two genotypes. In the WT, the most highly induced gene encoded for glutamate decarboxylase, an enzyme that produces γ-Aminobutyric acid (GABA). GABA is a compound that may have several roles in the response to stress such as regulation of the cellular pH, osmoregulation and/or protection against oxidative stress [103]. On the other hand, in the *Ljgln2-2* mutant the most up-regulated gene encoded for a thaumatin-like protein (probeset chr1.CM0012.67). Thaumatin-like proteins normally accumulate as a consequence of pathogens attack and exert an antibacterial/antifungal effect [104], but are also produced as a consequence of abiotic stress. Genes involved in proline metabolism were also induced in both genotypes [100], but were not present among the 10 top up-regulated ones.

Drought stress induced the modulation of genes related to cell wall metabolism. A gene encoding for pectinesterase, an enzyme involved in cell wall catabolism, was highly induced in both genotypes (probeset Ljwgs_036708.1). Moreover, a gene encoding for expansin, an enzyme involved in cell-wall loosening during the enlargement of plant cells, was highly down-regulated in the WT (probeset Ljwgs_056053.1). Taken together these results indicate that both WT and mutant genotypes are undergoing restructuration of the cell wall under conditions of water deprivation.

WT			
Probeset	**log_2 FC**	**Description**	**Similar to**
Up-Regulated			
TC11101	4.83	Glutamate decarboxylase	AT5G17330
chr1.TM1635.18	4.77	ACC synthase	AT3G61510
Ljwgs_047159.1	4.61	STIG1-related protein	AT1G11925
chr5.CM0909.59	4.47	Glutathione S-transferase	AT2G29420
chr1.CM0141.2	4.37	Nitrate / peptide transporter	AT1G32450
Ljwgs_036708.1	4.32	Pectinesterase	AT2G45220
chr4.CM0429.5	4.32	Mitochondrial inner membr. translocase	AT4G16160

		Inositol-1,4,5-trisphosphate 5-	AT1G47510
chr5.CM0089.120	4.15	phosphatase	
chr5.CM0148.50.2	4.11	Cytochrome P450	AT5G52400
TM0763.11	4.08	12-oxophytodienoate reductase	AT2G06050
Down-regulated			
Ljwgs_040576.1	−4.15	Glutaredoxin	AT5G18600
Ljwgs_056053.1	−4.15	Alpha-expansin family protein	AT2G39700
Ljwgs_006332.1	−3.92	Hypothetical protein	AT1G30260
chr1.BM1732.18	−3.82	Hypothetical protein	AT3G11210
BM0976.11	−3.78	Hypothetical protein	AT2G01050

Table 5. Top 10 genes up- or down-regulated by drought in WT and *Ljgln2-2*.

WT			
Ljwgs_028040.1	−3.74	Ammonium transporter	AT4G13510
chr1.CM0233.42	−3.47	Nucleic acid binding protein	AT1G52950
		hydroxycinnamoyl-CoA	AT2G19070
		shikimate/quinate	
chr6.TM1374.27	−3.41	hydroxycinnamoyltransferase-like	
Ljwgs_016759.2	−3.41	Chloride channel protein	AT5G40890
TM1490.11	−3.35	MYB transcription factor	AT2G21650
Ljgln2-2			
Probeset	**log_2 FC**	**Description**	**Similar to**
Up-Regulated			
chr1.CM0012.67	8.20	Thaumatin-like protein	AT1G20030
Ljwgs_075692.1.1	7.87	GDSL esterase/lipase	AT1G29660
chr3.TM1465.12	7.50	SPX domain-containing protein	AT2G45130
chr1.TM1635.18	7.34	ACC synthase	AT3G61510
Ljwgs_036708.1	6.93	Pectinesterase	AT2G45220
chr2.CM1150.57	6.84	Metalloendoproteinase	AT1G70170
chr1.CM0104.32	6.84	NAC domain transcription factor	AT3G04070
Ljwgs_020980.2	6.80	C2H2 transcription factor	AT2G37430
Ljwgs_047159.1	6.73	STIG1-related protein	AT1G11925
		Late embryogenesis abundant	AT3G53040
Ljwgs_145133.1	6.72	(LEA)	
Down-regulated			
chr1.BM1732.18	−8.81	Lipase/hydrolase protein	AT3G11210
chr2.CM0249.113	−7.27	Myb familily transcription factor	AT2G19510
Ljwgs_040576.1	−6.77	Glutaredoxin	AT5G18600
Ljwgs_073999.0.1	−6.70	Cytochrome P450	AT1G24180

Ljwgs_052903.1	−6.12	Lipoxygenase	AT1G55020
Ljwgs_058749.1	−5.73	Beta-glucosidase	AT5G42260
Ljwgs_127990.1	−5.62	Myb-related transcription factor	AT4G39250
TM0990.31.1	−5.59	Hypothetical protein	-
chr3.CM0590.43	−5.55	Hypothetical protein	AT4G27450
Ljwgs_091781.1	−5.45	Lipoxygenase	AT1G55020

Table 5. *Cont.*The table shows the probesets corresponding to the 10 genes that showed higher extent of modulation (FDR < 0.05) in their transcript levels in the leaves of *L. japonicus* plants after 4 days of water deprivation as determined by transcriptome analysis using Affymetrix genechips. The $\log_2$ of the fold-change is indicated together with the description of the most probable match and the most similar Arabidopsis gene. The following databases were used for the search: the Kazusa DNA research institute (Available online: http://www.kazusa.or.jp/lotus/blast.html; accessed on March 16, 2012), Non-redundant protein sequences (Available online: http://blast.ncbi.nlm.nih.gov/; accessed on March 16, 2012), TAIR (Available online: www.arabidopsis.org; accessed on March 16, 2012) and the Legume transcription factor database (Available online: http://legumetfdb.psc.riken.jp/index.pl; accessed on March 16, 2012).

Several transcription factors (TFs) and genes related to signal transduction were among the most regulated by drought. Up-regulation of genes encoding for putative TFs was seen only in *Ljgln2-2*, where a NAC domain and a C_2H_2 family TFs were highly induced under drought conditions (probesets chr1.CM0104.32 and Ljwgs_020980.2). Since much more genes were modulated by drought in *Ljgln2-2* compared to the WT, these TFs may be involved in the unique transcriptional response of the mutant. An inositol-1,4,5-trisphosphate 5-phosphatase and a SYG1/Pho81/XPR1 (SPX) domain containing protein, both related to signal transduction, were induced in the WT and mutant respectively. Inositol-1,4,5-triphosphate is one of the major phospholipid-derived signaling molecule involved in signal transduction following osmotic stress [97].

Finally, other highly modulated genes encoded for redox-related enzymes like glutathione-S-transferase (probeset chr5.CM0909.59, induced in the WT) and glutaredoxin (probeset Ljwgs_040576.1). The gene coding for glutaredoxin was the first and third most repressed one in WT and *Ljgln2-2* respectively. Glutaredoxins are glutathione-dependent oxidoreductases that can regulate enzyme activity through reduction of enzyme disulfide bridges, and plays a crucial role in plant development and in the response to oxidative stress [105].

It is interesting to notice that the average fold-change of both up- and down-regulated genes was much higher in the case of the mutant (Table 5). This was also confirmed at the whole-transcriptome level [100]. This behavior

indicates that the *Ljgln2-2* mutant may be suffering or perceiving a higher level of cellular stress than the WT after similar conditions of drought-stress. Besides of these differences, several of the top-modulated genes in WT and *Ljgln2-2* were related to similar functions like hormone metabolism, cell-wall metabolism and redox metabolism. This indicates that the modulation of these metabolic processes is part of the core response of *L. japonicus* to water deprivation and is independent from the presence of plastidic GS.

In conclusion, the analysis carried out here indicated that during water deprivation in *L. japonicus* a common response is triggered in both WT and *Ljgln2-2* including modulation of genes for hormone, cell-wall and redox metabolism as well as TFs and genes involved in signal transduction. However, much more genes were modulated in the mutant plants, and the extent of their modulation was more pronounced, reflecting the importance of plastidic GS in the response of the plant to drought stress. Some TFs were highly induced exclusively in the mutant, suggesting their possible involvement in the particular transcriptional response of the mutant to drought stress. The development of a TILLING reverse genetic tool [106] and, much more recently, of a population of insertion mutants created using the LORE1 endogenous retrotransposon [107] in *L. japonicus* may permit in the near future the evaluation of mutants of highly drought-responsive genes in this model legume.

2.2.5. Co-Expression Analysis of *L. japonicus* Plastidic GS

The increasing amount of plant transcriptomic data available in public databases has boosted the number of studies that explore gene function using *in silico* approaches. An example of this consists in data mining for genes that have similar expression profiles (also called co-expression) under a large number of conditions. Such kind of analysis is of particular interest since it may reveal novel interconnections between different metabolisms in virtue of a similar expression of their genes. Based on the assumption that genes sharing the same expression profile are likely to be functionally related (the so called “guilt by association”), many genes have been associated in a wide range of organisms, including plants. The analysis of gene co-expression requires the availability of a large amount of high-quality data, generally from DNA microarrays under different experimental conditions. For this reason, co-expression studies have been carried out only in a limited number of model plants like *Arabidopsis*, barley, rice and poplar [108,109]. However, an online tool for co-expression analysis has been made available recently for *L. japonicus* (“The Lotus transcript profiling resource”, available online: http://cgi-www.cs.au.dk/cgi-compbio/Niels/index.cgi; accessed on March 24, 2012). This program utilizes the

dataset from different arrays published by Høgslund *et al.* [110] that includes transcriptomic data from different tissues of WT and mutant *L. japonicus* plants.

Using this resource, the top 100 genes whose transcription was positively correlated with the plastidic GS2 were identified. A Pearson distance cut-off value of <0.3 was applied in order to consider only highly positively co-expressed genes. This dataset was analyzed using the gene expression analysis feature of Genebins [111], a program that permits to detect if a certain functional category of genes (also called "BIN") is statistically over-represented within a group. Using the default parameters given by the program ($p < 0.05$, Bonferroni correction), it was found that the functional category "starch synthase" was statistically ($p = 3.8 \times 10^{-9}$) more represented between this group of genes. This strong correlation of plastidic GS2 with starch metabolism was evident at a glance from the overview of general metabolism given by the MapMan program, where a total of seven probesets were assigned to the starch/sucrose pathway (five corresponding to starch synthase and two to beta amylase), with only six more probesets divided between the other metabolic routes (Figure 4). These results confirmed once again that plastidic GS2 is involved in the carbon metabolism of *L. japonicus* and, more specifically, suggested an involvement of this isoform in the starch and sucrose metabolism. This has been confirmed experimentally using the mutant *Ljgln2-2*, that showed substantially lower levels of starch and sucrose in the nodules than the WT affecting nodulation and nitrogen fixation [81] (see also Section 2.2.3). It has to be noticed that the transcriptomic dataset used to obtain the co-expression data presented here were mainly from non-photosynthetic tissues [110]. It may be then possible that the association of plastidic GS2 with starch and sucrose metabolism detected by the present study may be specific of roots and nodules. While plastidic GS2 is predominantly expressed in the green tissues of most plants, the presence of the GS2 polypeptide in non-photosynthetic tissues seems a typical feature of temperate legumes [25]. Nevertheless, a recent study correlated quite interestingly the rice plastidic GS2 gene expression with genes coding for the structural proteins of the photosystems and the enzymes of Calvin cycle and photorespiration [109].

Finally, we have to say that of the top 100 genes that were positively correlated with plastidic GS2 in co-expression analysis, 71 were defined either as unclassified with homologue or unclassified without homologue according to the KEGG-based ontology of Genebins, and 8 of them were involved in transcriptional regulation. Further work is still required to fully characterize the whole network of proteins that may be associated with plastidic GS2. As it has

been reviewed in this paper, the plastidic GS2 from *L. japonicus* is clearly involved in many distinct processes. The everyday increasing amount of transcriptomic data, bioinformatics tools and mutant collections now available in *L. japonicus* will enable in the near future to better define the precise involvement of this important protein in the metabolic regulation of legume plants.

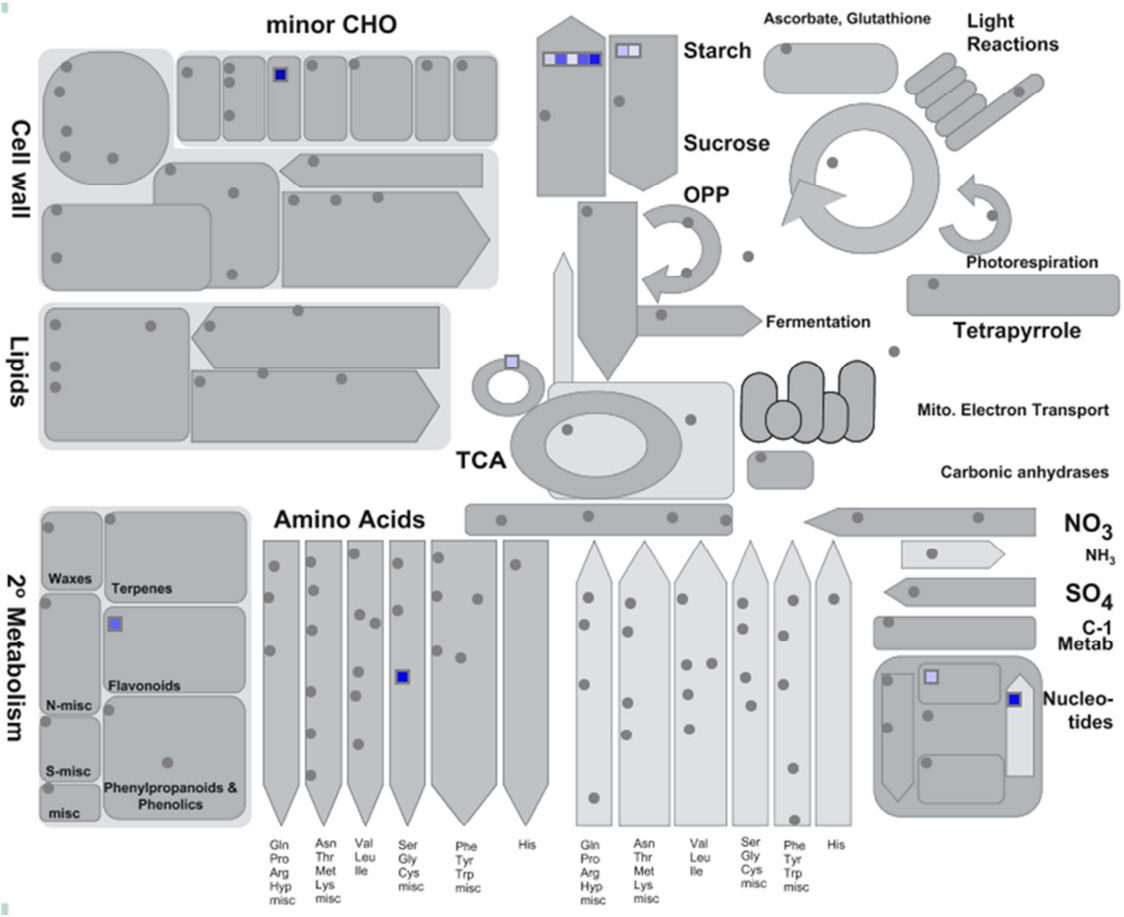

Figure 4. Co-expression analysis of plastidic GS. An overview of general metabolism was created using MapMan. Blue squares represent genes that are positively co-expressed (Pearson distance <0.3) with plastidic GS. The intensity of the blue color is proportional to the degree of correlation between a certain gene and the plastidic GS gene.

2.2.6. Analysis of Heterozygous Plastid GS Mutant Plants

We have shown above that homozygous photorespiratory mutants affected in plastidic GS have been of fundamental importance to study plastidic GS functionality in *L. japonicus* plants as a result of of plastidic GS impairment. We have also analyzed the phenotypic behavior of *Ljgln2-1* and *Ljgln2-2* GS heterozygous mutant plants. While, in the long term, the homozygous photorespiratory mutants were not viable at ambient CO_2 concentrations,

heterozygous *Ljgln2-1* and *Ljgln2-2* mutants could be grown normally in air without showing any apparent stress symptom or ammonium accumulation, thus confirming the recessive character of the mutant traits. Quantification of the GS protein and enzyme activity levels in the heterozygotes led to quite interesting findings. It was observed that heterozygote *Ljgln2-2* had intermediate levels of total GS activity and plastidic GS protein, as it was expected according to the behavior of similar plastidic GS mutants from barley [23]. In contrast, the heterozygote *Ljgln2-1* showed normal levels of plastidic GS polypeptide and cytosolic GS, but completely lacked of plastidic GS activity, similarly to the homozygous mutant plants. This interesting behavior indicated that *Ljgln2-1* mutation is recessive for the air-sensitivity, ammonium accumulation and plastidic GS polypeptide level phenotypes, but it is a negative dominant mutation in regard to plastidic GS activity. This means that the presence of inactive mutant subunits of plastidic GS is able to avoid the formation of active GS oligomers even in the presence of wild-type active subunits of plastidic GS.

The concept of negative dominant mutation was first defined by Herskowitz [112] on its classic paper in *Nature*. Curiously, in this paper it was predicted that the GS enzyme, in virtue of its multimeric nature, might be particularly sensitive to the presence of negative dominant mutations in the GS genes. However, how can be explained the air-insensitivity phenotype showed by heterozygous *Ljgln2-1*, which has normal levels of the GS2 polypeptide but apparently no GS2 activity? One possible explanation may be that heterozygous *Ljgln2-1* has almost undetectable levels of GS2 activity, but some tiny residual activity that could be sufficient to efficiently reassimilate the ammonium produced by photorespiration. This hypothesis implies that plastidic GS is present in a large excess in the chloroplasts of *L. japonicus*. Considering that photorespiration is a high-flux pathway [73], and that the ammonium assimilated through photorespiration may be 10 times higher that the one trough primary assimilation [4], it seems improbable that a residual amount of activity may be sufficient to reassimilate all the ammonium produced by glycine decarboxylation when the heterozygous *Ljgln2-1* plants are grown under normal air conditions. An alternative and more likely explanation may be that in the heterozygote *Ljgln2-1* plants different types of WT-mutant hetero-oligomers may be predominantly formed that could be at least partially active *in vivo*, but their GS enzyme activity may be lost when extracted from the cell in order to obtain crude extracts for the determination of the *in vitro* GS activity. It is possible that some post-translational modification of Lotus plastidic GS is essentially required for enzyme activity *in vivo* and may be altered as a result of *Ljgln2-1* mutation when preparing the crude extracts from heterozygous plants.

3. CONCLUSIONS

In this paper we have described the recent progress made in our laboratory on the structure-function and functional genomics of glutamine synthetase, a crucial enzyme of nitrogen metabolism in legumes. Main advances produced can be summarized as follows: (1) We have shown that the quaternary structure of plant GS depends on metal cofactors and, in some cases, is highly unstable and can be disassembled by single-point mutations resulting in a complete loss of enzymatic activity; (2) We report the existence of different activity forms of plant GS with different affinity towards glutamate and other types of evidence which are all suggestive of post-translational modifications of this protein; (3) We also show the important significance of plastidic GS2 isoform in various processes such as photorespiration, nitrogen nutrition, nodulation, and drought-stress. In fact, it is described how the lack of GS2 produces major changes in the transcriptome of *L. japonicus* plants and, in many cases, changes in carbon metabolism.

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Publicación 7.

Reassimilation of Ammonium in *Lotus japonicus*

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Cf. Apartado "photorespiratory ammonium reassimilation"

ABSTRACT

This review summarizes the most recent results obtained in the analysis of two important metabolic pathways involved in the release of internal sources of ammonium in the model legume *Lotus japonicus*. On the one hand, photorespiratory metabolism, and, on the other hand, asparagine breakdown mediated by aparaginase. The use of photorespiratory mutants deficient in plastidic glutamine synthetase (GS2), enabled to investigate the transcriptomics and metabolomic changes associated to photorespiratory ammonium accumulation in this plant. The results obtained indicate the existence of a coordinate regulation of genes involved in photorespiratory metabolism. Other types of evidence illustrate the multiple interconnections existing among the photorespiratory pathway and other processes such as intermediate metabolism, nodule function and secondary metabolism in this plant, all of which are substantially affected in GS2-deficient mutants because of the impairment of the photorespiratory cycle. Finally, the importance of asparagine metabolism in *L. japonicus* is highlighted because of the fact that asparagine constitutes the vast majority of the reduced nitrogen translocated between different organs of this plant. The different types of asparaginase enzymes and genes which are present in *L. japonicus* are described. A particular focus is made on the most abundant K^+-dependent NSE1 isoform and how TILLING mutants were used to demonstrate by reverse genetics the importance of this particular isoform in plant growth and seed production.

INTRODUCTION

The use of nitrogen by plants involves several steps, including uptake, assimilation, translocation and different forms of recycling and remobilization processes, all of them of crucial importance in terms of nitrogen utilization efficiency (Hirel *et al.*, 2007; Márquez *et al.*, 2005; Masclaux-Daubresse *et al.*, 2010; Tabuchi *et al.*, 2007). Ammonium can be produced in plants as a result of nitrate reduction by external primary nitrogen assimilation. Alternatively, ammonium can be also produced in the nodules of particular plant species such as legumes as a result of the action of biological dinitrogen fixation by symbiosis with bacteria. In addition, other processes exist in the plants which give rise to the production of endogenous sources of ammonium which has to be efficiently reassimilated. These latter processes are in general called secondary ammonium assimilation and include photorespiration and other catabolic processes such as phenylpropanoid, ureide or amino acid catabolisms (Joy, 1988). In this paper the attention will be focused mostly on the photorespiration and asparagine catabolic processes.

Photorespiration is the most important process in which high amounts of ammonium are released at a rate that can exceed by 10-fold the rate of primary nitrate assimilation in plants (Keys *et al.*, 1978). Ammonium is produced as a result of the decarboxylation of two molecules of glycine to yield one molecule of serine in a reaction catalyzed by glycine decarboxylase (GDC, EC 2.1.2.10) in the mitochondria (Fig. 1). This reaction forms part of a more complex process called as the photorespiratory nitrogen cycle. This cycle is also called C2 cycle because it starts by means of the oxygenase activity of RUBISCO in the chloroplasts, which produces 2-phosphoglycolate (a 2C compound), and is aimed to retrieve 3 out of each 4 carbon atoms entering this pathway as 2-phosphoglycolate in order to yield 3-phosphoglycerate which goes back to the Calvin cycle (Bauwe *et al.*, 2010; Keys *et al.*, 1978; Maurino and Peterhansel, 2010; Wingler *et al.*, 2000). The evidence presented by Keys *et al.* (1978) in support of their original formulation of the photorespiratory N cycle consisted principally of data demonstrating that (1) although leaf mitochondria contained glutamate dehydrogenase (GDH, EC 1.4.1.2), a potential ammonium assimilator, they could not reassimilate ammonium; and (2) the N-assimilating enzyme appeared to be glutamine synthetase (GS, EC 6.3.1.2), a conclusion supported by the finding that the GS-inhibitor methionine sulfoximine (MSX) blocked reassimilation of ammonium and caused it to accumulate rapidly and in quantity (Givan *et al.*, 1988; Keys, 2006).

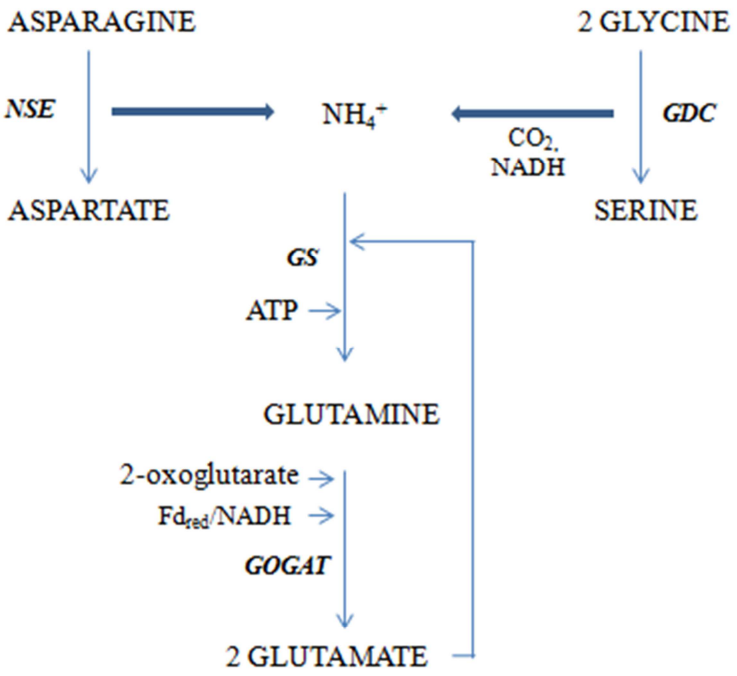

Fig. 1. Asparaginase (NSE) and glycine decarboxylase (GDC) reactions constitute two major sources of ammonium in legume plants such as *Lotus japonicus*. Ammonium produced has to be effciently reassimilated by means of the glutamine synthetase (GS) / glutamate synthase (GOGAT) cycle. Reduced ferredoxin (Fd_{red}) or NADH can be the electron donors for the GOGAT reaction.

Although it was initially thought that cytosolic glutamine synthetase (GS1) could be in charge of photorespiratory ammonium assimilation, it was later demonstrated that it was the chloroplastic form of glutamine synthetase (GS2) the one responsible for this process, as a result of the isolation of photorespiratory mutants in barley which were specifically deficient in plastidic GS (Wallsgrove *et al*., 1987; Keys, 2006). Interestingly, no such mutants could be found in the exhaustive screening for photorespiratory mutants previously carried out in *Arabidopsis*, while it was possible to identify mutants impaired in most of the steps of the C2 photorespiratory nitrogen cycle (Somerville and Ogren, 1979; see also Timm and Bauwe, 2013 for a recent update). An ample set of photorespiratory mutants have been described from other plant species, such as barley and pea (Blackwell *et al*., 1988; Keys and Leegood, 2002). The first GS photorespiratory mutants isolated from legume plants were identified several years ago in our laboratory from the model legume *Lotus japonicus* (Orea *et al.*, 2002; Márquez *et al*., 2005). These mutants were shown to be specifically deficient in GS2 and have been substantially characterized at the molecular and physiological levels (Orea *et al.*, 2002; Márquez *et al.*, 2005; Betti *et al*., 2006; Díaz *et al*., 2010; García-Calderón *et al.*, 2012; Betti *et al.*, 2012; Pérez-Delgado

et al., 2013). The work recently carried out with these mutants will be summarized in the first part of this article.

Another important source for endogenous ammonium in plants, particularly in temperate legumes, is the breakdown of asparagine catalyzed by asparaginase (Fig. 1), which constitutes the focus of the second part of this article. Asparagine has a N:C ratio of 2:4 (higher than glutamine), it is very stable and soluble and has high mobility at physiological pH, which makes it an efficient molecule to store and transport reduced nitrogen between plant tissues. In fact, asparagine is the major transport compound in the xylem from the root to the leaves and in the phloem from the leaves to the developing seeds in a range of plants, particularly in temperate legumes (Andrews, 1986; Lea *et al*., 2007*a*). Asparagine also plays an important role in the regulation of N flux in the N-organic pool (Lam *et al*., 1996) and stress responses (Lea *et al.*, 2007*a*). The importance of asparagine in legume plants has been also pointed out by a possible involvement of asparagine in the cycling of amino acids between the bacteroid and the plant (Prell and Poole, 2006). In fact, nodule-specific modulation of GS in transgenic *Medicago truncatula* leads to inverse alteration in asparagine synthetase expression (Carvalho *et al.*, 2003). In addition, it is also known that the high free asparagine trait may represent a physiological marker associated with high seed protein concentration. Free asparagine may also act as a metabolite signal in the developing embryo and influence seed protein accumulation (Pandurangan *et al*., 2012*)*. In the case of the model legume *L. japonicus*, it was shown that asparagine metabolism must be of crucial importance since asparagine can account for 86% of the nitrogen flux from root to shoot, when the plant is adequately fed (Waterhouse *et al.*, 1996).

Asparagine levels are tightly regulated through a control of its metabolism and its intracellular concentration due to a balance between biosynthesis and degradation. The main route for asparagine biosynthesis in plants is mediated by the enzyme asparagine synthetase (AS, EC 6.3.5.4), which catalyzes the ATP-dependent transfer of the amido group of glutamine (or in minor proportion ammonium) to aspartate producing asparagine, glutamate, AMP and PPi in the presence of Mg^{2+} (Lea *et al*., 2007*a*) (Fig. 2). On the other side, degradation of asparagine can be produced by two alternative ways based either on deamination or transamination reactions. Transamination reactions from asparagine to glyoxylate yield 2-oxosuccinamic acid and glycine, in a reaction catalyzed by serine-glyoxylate transaminase (SGAT, EC 2.6.1.45), which has been shown to have dual specificity for both serine and asparagine (Zhang *et al.*, 2013). This route probably has a small contribution to net asparagine catabolism, since this

transamination reaction probably takes place in the context of the photorespiratory cycle, where, as mentioned above, the majority of nitrogen is continuously recycled (Lea *et al.*, 2007*a*).

Other transaminations in minor proportion from asparagine to pyruvate, oxalacetate or 2-oxoglutarate can also exist (Ireland and Joy, 1983*a*). However, the main route for asparagine degradation is through deamidation and takes places mainly in developmental tissues, where an active protein synthesis is needed in order to provide amino acids of the aspartate family, by the action of asparaginase. Asparaginase (NSE, EC 3.5.1.1) catalyzes the hydrolysis of asparagine to aspartic acid and ammonium (Fig. 2), which is subsequently reassimilated for the biosynthesis of other nitrogen compounds (Cánovas *et al.*, 2007; Lea *et al.*, 2007*a*). The main results found on the structure and function and biological significance of asparaginases in *L. japonicus* are summarized in the second part of this article.

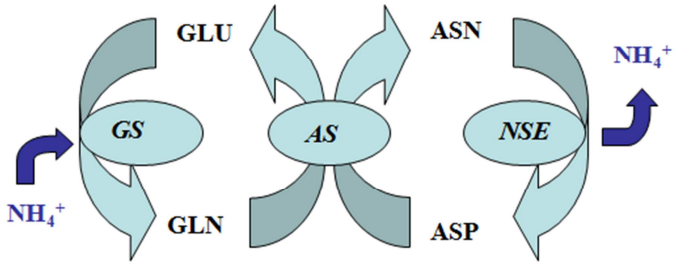

Fig. 2. Biosynthesis and degradation of asparagine which is the principal molecule used to transport reduced nitrogen in *L. japonicus* plants. Ammonium is first assimilated into the amido group of glutamine by the glutamine synthetase (GS) reaction, and then the amido group of glutamine is transferred to aspartate to yield asparagine by the reaction catalyzed by asparagine synthetase (AS). Later on, the asparaginase reaction (NSE) releases ammonium from the asparagine breakdown.

Reassimilation of photorespiratory ammonium in *L. japonicus*

Photorespiratory ammonium accumulation in mutants deficient in plastidic GS (GS2).

Two *L. japonicus* photorespiratory mutants deficient in GS2 but having normal levels of GS1 were identified in our laboratory which were initially called *Ljpr1* and *Ljpr2* (Orea *et al.*, 2002; Márquez *et al.*, 2005). These mutants were allelic and showed a Mendelian inheritance of a single recessive trait (Orea *et al.*,

2002). Later on it was shown that both mutants were affected in the coding region of the *LjGLN2* gene and were consequently denominated as *Ljgln2-1* and *Ljgln2-2* respectively (Betti *et al*., 2006). A single-point allelic mutation was identified on each of these mutants that was responsible for two different amino acid replacements (G85R and L278H, respectively). Both mutations affected the stability of the GS2 polypeptide, although in a different manner. Small levels of GS2 proteins could be detected in leaves, roots and nodules of *Ljgln2-1* mutant, but a complete lack of GS2 protein was observed in *Ljgln2-2* (Betti *et al*., 2006; García-Calderón *et al*., 2012).

Ljgln2-1 and *Ljgln2-2* mutants are able to grow in a similar manner than the wild type (WT) on a high CO_2 atmosphere (0.7% v/v) (photorespiration suppressed conditions) but showed an air-sensitive phenotype when the plants are transferred to a normal air atmosphere (photorespiratory active conditions). Typical air-sensitive symptoms of these photorespiratory mutants are chlorosis and necrosis of the edges of some young leaves, which turned yellow and warped after 1-2 weeks in air. Another characteristic of GS2-mutants is the fact that they accumulate high levels of ammonium when transferred from high CO_2 to air conditions (Orea *et al*., 2002), as it was also shown previously in barley (Wallsgrove *et al.*, 1987) and in GS2-antisense oilseed rape lines (Husted *et al.*, 2002). These results confirm the essential role of GS2 for the reassimilation of photorespiratory ammonium, a process that cannot be assumed by GS1. However, it was recently shown in *L. japonicus* GS2-minus mutants that this increase in photorespiratory ammonium took place gradually up to three days of the transfer from high CO_2 to air conditions, reaching a maximum, and then there was a gradual decline in the ammonium content reaching a minimum after 8-10 days of transfer to air (Pérez-Delgado *et al.*, 2013; see also Table 1). The sections below summarize the main experiments carried out to give some possible explanations for this drop in ammonium content produced in *L. japonicus* GS2 mutants after three days of active photorespiration.

Implications in the regulation of genes for photorespiratory metabolism and other related genes

The levels of expression of *L. japonicus* photorespiratory genes were determined by quantitative real-time (qRT-PCR) analysis both in WT and *Ljgln2-2* photorespiratory mutants.

time (days of transfer to air)	Ammonium content (µmol g^{-1} FW)	
	WT	***Ljgln2-2***
0	0	1.28 ± 0.10
3	0.44 ± 0.04	56.69 ± 10.87
6	0.58 ± 0.16	29.19 ± 2.42
10	1.64 ± 0.18	27.96 ± 2.74

Table 1. Accumulation of ammonium in WT or *Ljgln2-2* photorespiratory mutants at different times of transfer from high CO_2 to normal air conditions. Ammonium measurements were carried out in leaves extracts as described by Orea *et al*. (2002). The average of two independent experiments with three biological replicates is shown.

It was very interesting to notice that most of the photorespiratory genes showed a common down-regulation in the mutant after two days of the transfer from high CO_2 to air conditions (Pérez-Delgado *et al.*, 2013). The results obtained are suggestive of the existence of a coordinate regulation of photorespiratory metabolism which may be produced as a way to avoid further accumulation of ammonium in *L. japonicus* GS2-deficient mutants. The fact that ammonium accumulation is highly toxic in plants (Britto and Kronzucker, 2002) can account for this regulation. In addition, the drop in ammonium content observed in these mutants after 3 days of transfer from high CO_2 to air conditions could be also explained by the fact that the levels of transcripts for *LjGLN1.2*, one particular gene encoding for cytosolic GS1, were significantly increased in the mutants, which was paralleled by a substantial increase in the levels of GS enzymatic activity and glutamine content of the mutant plants (Pérez-Delgado *et al.*, 2013). Other qRT-PCR measurements have shown that this up-regulation of cytosolic GS1 was also accompanied by an up-regulation in the levels of transcripts for other ammonium assimilatory genes (Pérez-Delgado *et al.*, unpublished). The analysis of the transcriptomes of WT and *Ljgln2-2* mutant plants confirmed also all these results. Fig. 3 shows a clustering of the transcript levels obtained for different genes which further illustrates on the down-regulation of photorespiratory genes and the up-regulation of transcripts for other ammonium assimilatory enzymes such as cytosolic GS1 and GDH.

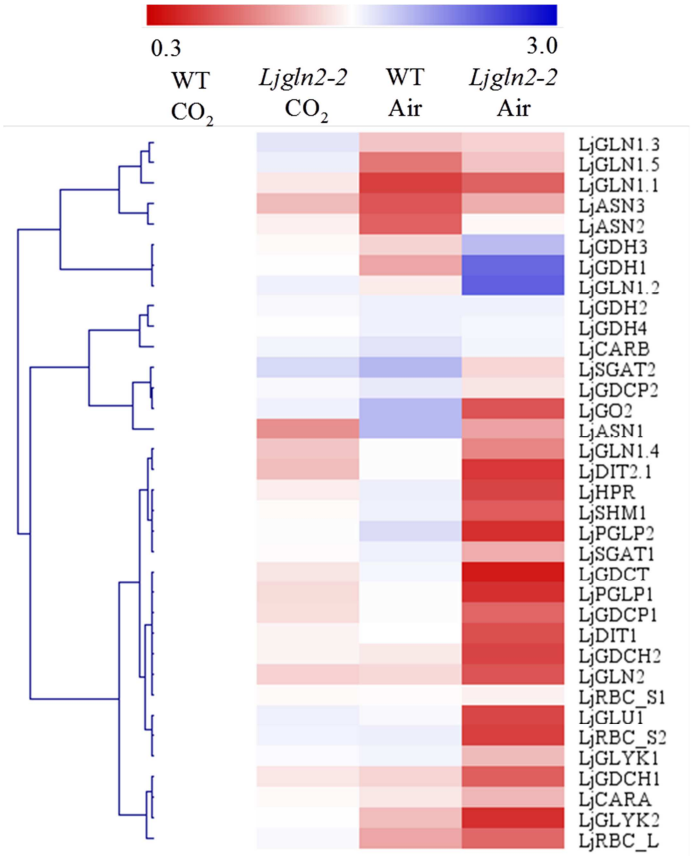

Fig. 3. Hierarchical clustering of transcriptomic data for some genes involved in ammonium assimilation and photorespiration. Transcript levels were determined for wild type (WT) and *Ljgln2-2* mutant plants grown in high CO_2 (photorespiration suppressed conditions) and after 2 days of transfer to normal air (photorespiration active conditions). The data were obtained from Affymetrix Gen Chip hybridizations carried out as described by Pérez-Delgado *et al*. (2013) using leaf RNA samples. For comparative purposes, the transcript levels in WT plants under high CO_2 conditions were taken as 1. Red and blue indicate lower and higher expression than in the WT high CO_2 controls, respectively. The color intensity represents the fold-change as indicated in the scale bar. The genes considered were: Rubisco (*LjRBC_L* , *LjRBC_S1* and *LjRBC_S2*), Phosphoglycolate Phosphatase (*LjPGLP1* and *LjPGLP2*), Glycolate Oxidase (*LjGO2*), Serine:Glyoxylate Aminotransferase (*LjSGAT1* and *LjSGAT2*), Glycine Decarboxylase H subunit (*LjGDCH1* and *LjGDCH2*), Glycine Decarboxylase P subunit (*LjGDCP1* and *LjGDCP2*), Glycine Decarboxylase T subunit (*LjGDCT*), Serine Hydroxymethyltransferase (*LjSHM1*), Hydroxypyruvate Reductase (*LjHPR*), Glycerate Kinase (*LjGLYK1* and *LjGLYK2*), Plastidic Glutamine Synthetase (*LjGLN2*), Ferredoxin-dependent GOGAT (*LjGLU1*), Plastidic Dicarboxylate Transporter (*LjDIT1* and *LjDIT2.1*), Cytosolic Glutamine Synthetase (*LjGLN1.1*, *LjGLN1.2*, *LjGLN1.3*, *LjGLN1.4* and *LjGLN1.5*), Glutamate Dehydrogenase (*LjGDH1*, *LjGDH2*, *LjGDH3*, *LjGDH4*), Asparagine Synthetase (*LjASN1*, *LjASN2* and *LjASN3*) and Carbamoyl Phosphate Synthetase (*LjCARA* and *LjCARB*). Hierarchical clustering of these data was performed using the Multiexperiment Viewer software version 4.8.1 (Saeed *et al*., 2006) with optimized gene leaf order and complete linkage clustering algorithm.

This induction of alternative ammonium assimilatory enzymes like GS1, concomitantly with the drop in ammonium accumulation observed in *L.*

japonicus mutant plants, is very interesting since it was shown that ectopic overexpression of GS1 in tobacco leaves was able to produce a 6- to 7-fold decrease in the levels of free ammonium (Oliveira *et al.*, 2002).

Implications in primary metabolism

The global analysis of the transcriptome of WT and *Ljgln2-2* mutant plants, both in high-CO_2 and air conditions, indicated that the aforementioned transcriptional changes were part of a vast modulation of the transcriptome caused by active photorespiration. The total number of genes modulated by the shift from high CO_2 to air conditions was much higher in the mutant plants: 6,610 probesets were changed in the *Ljgln2-2* genotype compared with 1,480 of the WT. The two genotypes shared 825 of these modulated probesets, while 655 and 5,785 probesets were modulated specifically in the WT or in the mutant plants, respectively (Pérez-Delgado *et al.*, 2013). These different numbers of probesets were further analyzed separately in order to investigate: a) the core/common response of *L. japonicus* to the change from high CO_2 to normal air, which is independent of GS2 (shared genes modulated both in WT and mutant plants); b) the normal response of the *L. japonicus* plants to active photorespiration that is directly or indirectly dependent on GS2 (genes modulated only in the WT); c) genes elicited by the presence of an impaired photorespiratory cycle and/or to the high levels of ammonium accumulated as a result of GS2-deficiency (genes modulated only in *Ljgln2-2* mutant plants). In this latter case, the analysis of the genes specifically elicited in *Ljgln2-2* detected more than 120 overrepresented gene families, spanning the majority of the primary and secondary metabolism, including, for example, carbon and nitrogen metabolism, biosynthesis of chlorophyll, amino acids metabolism, glycolysis, the Krebs cycle, and starch/sucrose metabolism, among others. This indicates that impairment of the photorespiratory cycle and/or the subsequent accumulation of ammonium have a global effect on the metabolism of the leaf, revealing the crucial importance of the reassimilation of photorespiratory ammonium and the multiple metabolic interconnections of it.

Comparative metabolomic analysis of WT and *Ljgln2-2* mutant plants established five different patterns of metabolites: a) Metabolites that increased in the mutant upon transfer from high CO_2 to air conditions, with no change in the WT. This group included several amino acids (Gly, Leu, Ile, Val, Trp, Phe) and organic acids (malate, citrate, succinate, 2-oxoglutarate). b) Metabolites that increased in the mutant upon transfer to air conditions, but decreased in the WT, which included manose, glucose, glucose-6-phosphate and glutamine. c) Metabolites that showed a higher content in the mutant background under high

CO_2, which sometimes increased upon transfer to air only in the mutant. These included many unknown compounds and particular organic acids such as saccharic acid, gluconic acid, glucaric acid-1,4 lactone and others. d) and e) Metabolites that increased/decreased in the WT or mutant but showed lower content in the mutant background under high CO_2 conditions or behave sometimes transiently. These groups included metabolites such as proline, trehalose, putrescine, myoinositol, and also key metabolites linking nitrogen and carbon metabolism such as aspartate and glutamate. The first two groups suggest the existence of metabolic pathways that were secondarily elicited as a result of impairement of the photorespiratory cycle and/or GS2 deficiency. Group c suggests the existence of metabolic pathways in which plastidic GS2 is constitutively required for particular metabolic processes. The two last groups of metabolites suggest the existence of general metabolic pathways required for the acclimation of the changes induced by the transition from high CO_2 to air (Pérez-Delgado *et al*., 2013).

The increase observed for various amino acids exclusively in the *Ljgln2-2* mutant plants suggest that nitrogen metabolism was not a limiting factor for the mutant fitness but rather a means to ammonium detoxification (Pérez-Delgado *et al.*, 2013). In line with this idea, the levels of these amino acids paralleled the peak of ammonium at day 3 of transfer to air. The decrease of glutamate observed in the *Ljgln2-2* mutant under air conditions could be explained by the great accumulation of several amino acids that may drain the glutamate pool. Other results indicate that aspartate and asparagine may be used more extensively as amino donors in the mutant under non-photorespiratory conditions, which may be possibly related to the fact that asparagine constitutes most (86%) of the nitrogen translocated in *L. japonicus* plants.

Four organic acids that are also intermediates of the Krebs cycle were significantly altered in the *Ljgln2-2* mutant, indicating a relationship between plastidic GS2 and central carbon metabolism (Pérez-Delgado *et al.*, 2013). The accumulation of ammonium as well as the increase in ATP/ADP ratio and in NADH and NADPH levels in photorespiratory mutants is known to inhibit the Krebs cycle at the level of pyruvate dehydrogenase (Bykova *et al.*, 2005; Bauwe *et al.*, 2010). The qRT-PCR measurements carried out in *Ljgln2-2* mutants indicated a general transcriptional repression over time of pyruvate dehydrogenase. Taking this into consideration, it seems unlikely that the accumulation of Krebs cycle intermediates may be related to a specific regulation of the genes involved in this pathway. It seems more likely that the lack of a functional GS2/Fd-GOGAT cycle in the chloroplast of *Ljgln2-2* may be

responsible for the increase in the levels of 2-oxoglutarate that enters the Krebs cycle and may force a higher flux of metabolites throughout this route. Interestingly, rice mutants lacking a cytosolic GS1 isoform showed a decrease in Krebs cycle intermediates (Kusano *et al.*, 2011). Both types of results point out the central role of GS in coordinating carbon and nitrogen metabolism in plants. Co-expression studies also confirmed the clear association between plastidic GS2 and carbon metabolism in *L. japonicus* plants (Fig. 4) (see also Betti *et al.*, 2012).

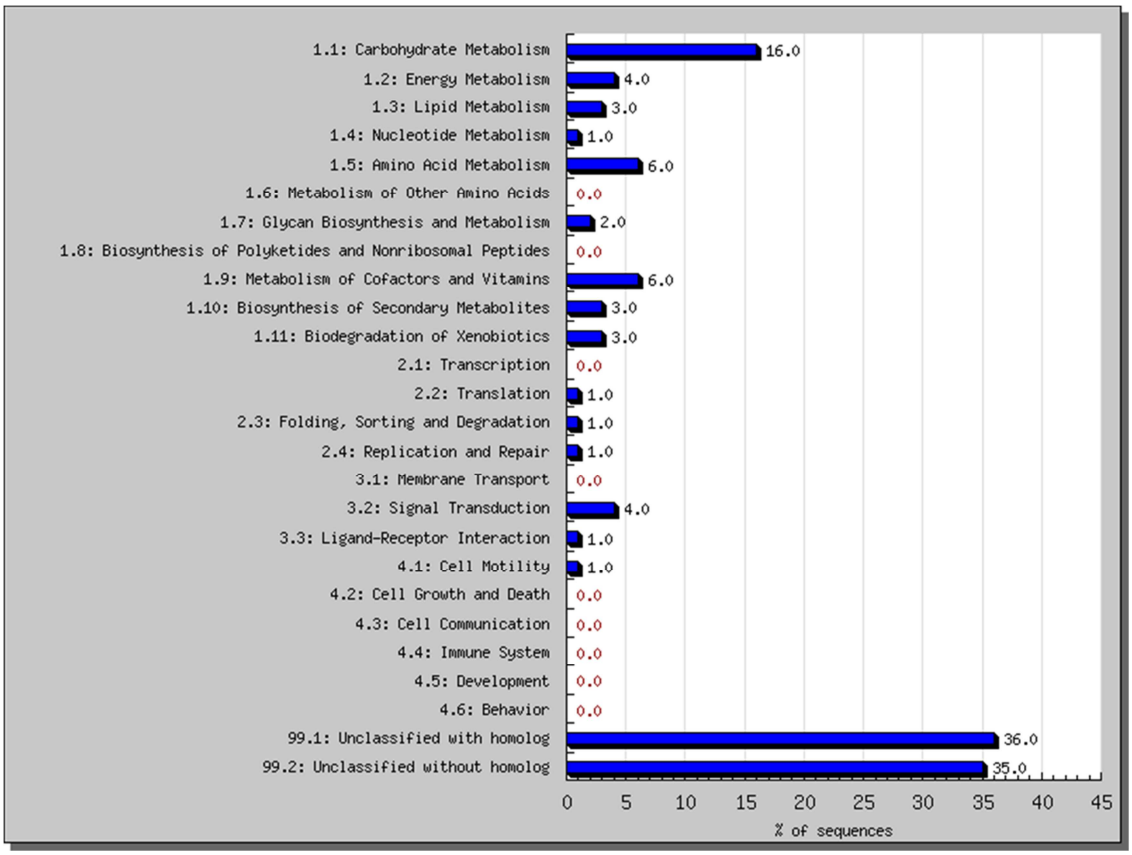

Fig. 4. Co-expression analysis of plastidic GS2. The list of the top 100 genes co-expressed together with *Ljgln2* was obtained at the "Lotus transcript profiling resource" (http://cgi-www.cs.au.dk/cgi-compbio/Niels/index.cgi) by searching in the profile matching tool with the probeset gi18266052 that corresponds to *Ljgln2*. A Pearson distance cut-off of <0.3 was applied in order to consider only highly co-expressed genes. These 100 genes were represented according to their gene ontology using the gene expression analysis tool of the GeneBins database (http://bioinfoserver.rsbs.anu.edu.au/utils/GeneBins-lotus/). The high number of genes for carbohydrate metabolism found in the group of co-expressed genes is highlighted in the Figure.

Implications for plant nodulation

The influence of photorespiratory metabolism and nodule function has been also examined in *L. japonicus* plants with the help of the photorespiratory mutants deficient in GS2 (García-Calderón *et al.*, 2012). It was established that when WT and mutant plants were transferred from high CO_2 to normal air conditions the nodulation process and nitrogen fixation were substantially affected, particularly in the mutant plants. This was attributed to a limitation in the carbon flux to the nodules due to the lower carbon availability and the onset of the photorespiratory activity of the plant. In fact, an important reduction in the levels of starch accumulation could be detected in the nodules of *L. japonicus* plants after transfer to air, becoming 50 % lower in the case of WT plants, and around 90 % lower in the case of the mutants. The levels of sucrose were also significantly decreased, particularly in the mutant plants. Optical microscopy studies revealed an anticipated senescence phenotype which was shown specifically the mutant plant nodules in air conditions and was also linked to a reduction in starch levels (García-Calderón *et al*., 2012). The fact that much more pronounced effects were detected in the mutant plants under active photorespiration conditions could be explained by the increased amount of non-assimilated ammonium representing a further inhibitory signal for the nodule formation pathway in agreement with other previous works that have recently shown that the nitrogen nutritional status of *L. japonicus* plants can strongly affect the competence for nodule formation (Omrane and Chiurazzi, 2009; Omrane *et al.*, 2009). Interestingly, several differences were also observed between the WT and GS2-deficient mutant plants even when the plants were maintained in high CO_2 conditions. In these conditions, a much higher decrease in starch, sucrose, glucose and fructose content was detected in the mutants, as well as some effects in nodulation and nitrogen fixation parameters (García-Calderón *et al.*, 2012). All these results would be in agreement with a major role of GS2 in the C/N balance of *L. japonicus* plants, as also concluded from transcriptomic and metabolomic studies mentioned above. Our results also emphasize how a "nitrogen" assimilation defect affects "carbon" metabolism and nodule function in *L. japonicus.*

Implications in secondary metabolism

Phenylpropanoids and their derivates are compounds produced from the amino acid phenylalanine and less frequently tyrosine, that have a wide range of bioactivity including auxin transport, antioxidant activity, pollen viability, plant pathogen and/or plant symbiont interactions (Buer *et al.*, 2010). In the past

decades, huge amount of new data was presented about genes, enzymes and transcriptional control of their biosynthetical pathways. The increasing knowledge can help to understand the importance of natural compounds in the interactions of plants with their environment (Petersen *et al*., 2010).

Both photorespiration and phenylpropanoid metabolisms are mainly related to carbon metabolism, but also to nitrogen metabolism, because of the use of amino acids as precursors. Therefore, the use of photorespiratory mutants could help to understand the cross-interaction between the two main processes of primary metabolism and the production of important secondary bioactive compounds.

Fig. 5 shows a Mapman overview of the main secondary metabolites pathways altered both in WT and *Ljgln2-2* mutants as a consequence of the transfer from high CO_2 to normal air conditions. It is easy to note that a much higher number of genes related to secondary metabolism are modulated in the *Ljgln2-2* mutant (Fig. 5B) compared to the WT (Fig. 5A), many of which were elicited exclusively in the mutant plants (Fig. 5C) in spite of the fact that some genes were jointly modulated both in the WT and mutants (Fig. 5D). Phenylpropanoid and flavonoids are among the most overrepresented metabolic pathways that are altered in *Ljgln2-2* mutant plants.

Phenylpropanoid and flavonoid biosynthesis in leaves of *L. japonicus* is generally stimulated in *Ljgln2-2* mutant after its transfer from elevated CO_2 where the rate of photorespiration is very low, to normal air. Firstly, there is valuable increase of the transcript *LjPAL1*, which encode the highly expressed isoform of phenylalanine ammonia lyase (EC 4.3.1.5) that was found to be responsive to environmental stimuli in *Arabidopsis* (Olsen *et al*., 2008). Deamination of phenylalanine by phenylalanine ammonia-lyase forms the phenylpropanoid skeleton, producing cinnamic acid which is the entry into the phenylpropanoid pathway.

Metabolite profiling showed an increased accumulation of several hydroxycinnamates in *Ljgln2-2* mutant, in comparison to the wild type (Pal'ove-Balang *et al.*, unpublished results). Several of those compounds, such as p-coumaric, ferulic and caffeic acid were found to have high antioxidant activity (Castelluccio *et al*., 1995; Rice Evans *et al.*, 1997), suggesting that there may be a higher requirement for this type of antioxidant compounds in the mutant plants due to the redox imbalance in active photorespiratory conditions.

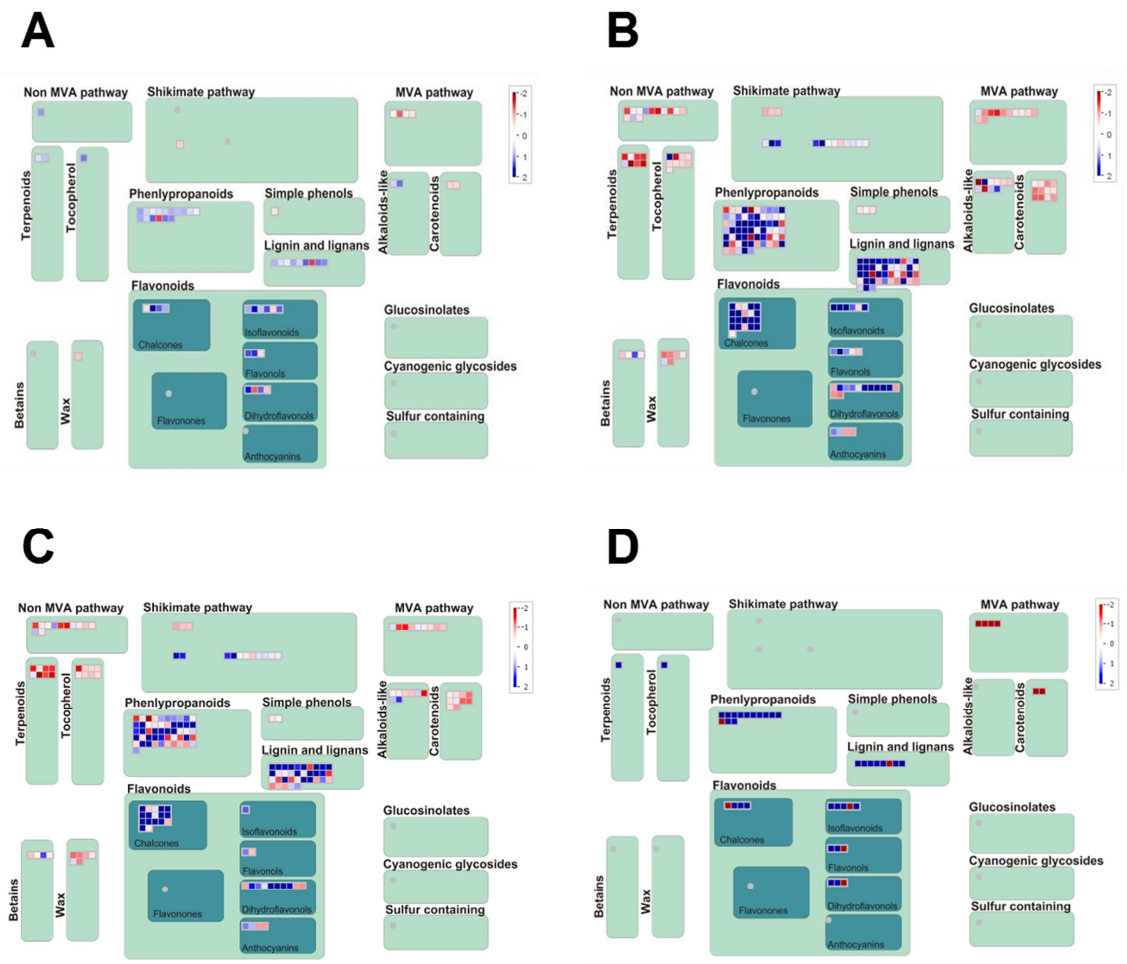

Fig. 5. MapMan overview of secondary metabolism in WT and *Ljgln2-2* plants after the re-activation of the photorespiratory cycle. 35 days-old plants were grown under CO_2-enriched atmosphere (suppressed photorespiration) and then transferred for 2 days to normal air conditions (active photorespiration) as described by Pérez-Delgado *et al*. (2013). The genes significantly ($p < 0.05$, FDR) modulated by active photorespiration in the leaves transcriptomes of WT and *Ljgln2-2* mutant plants are shown. Red and blue indicate lower and higher expression than in the high CO_2 controls, respectively. The color intensity represents the extent of modulation as indicated in $\log_2$ in the scale bar. (A) Genes modulated in the WT genotype. (B) Genes modulated in the *Ljgln2-2* mutant. (C) Genes modulated exclusively in the *Ljgln2-2* mutant. (D) Genes modulated in both genotypes. The genes shown in panel D changed in the same direction in WT and *Ljgln2-2*, but the extent of this change was different in the two genotypes. For this reason, a fold-change value of 2 was imposed for induced genes and of -2 for repressed genes.

The increase in *LjPAL1* expression in photorespiratory conditions is accompanied by the similar overexpression of *Lj4CL*, mainly the *Lj4CL3* gene that encodes for an enzyme which has a high affinity to *p*-coumarate and suggested to be important for flavonoid synthesis as previously reported (Costa *et al.*, 2005; Ehlting *et al.*, 1999). In contrast, other isoenzymes with high affinity to caffeic and/or ferulic acids were not markedly changed in photorespiratory conditions. Enhancement of flavonoid biosynthesis by increased transcription of

chalcone synthase (At5G13930) and chalcone isomerase (At3g55120) orthologs was found in *Ljgln2-2* and *Atglu1-2* photorespiratory mutants (Kissen *et al*., 2010; Pérez-Delgado *et al*., 2013). Moreover, the biosynthesis of tannins and anthocyanins can be stimulated by higher expression of dihydroflavonol-4-reductase gene. In agreement with this statement, it was observed that the *Lotus Ljgln2-2* photorespiratory mutant accumulated more tannins than the WT, whereas flavonols (mainly kaempferol) were generally lower in the mutant plants, in spite of the fact that the levels of these compounds were increased after transfer from high CO_2 to normal air (Pal'ove-Balang *et al*., unpublished results). In addition, the *Ljgln2-2* mutant also accumulates more isoflavonoids than the WT, in agreement with the higher expression observed for the chalcone synthase, vestitone reductase and isoflavone reductase genes.

Plant flavonoid compounds may confer various physiological functions for plants to survive and to adapt to environmental disturbances. The relatively high biosynthesis pattern of fenolic compounds in *Ljgln2-2* mutant on photorespiratory conditions, seems to be somewhat similar to changes in *Arabidopsis* due to different types of abiotic stress (Lillo *et al*., 2008), including nitrogen depletion. Several of the up-regulated genes are under control of PRODUCTION OF ANTHOCYANIN PIGMENT - PAP1/PAP2 transcription factors from the R2R3MYB family, which were found to be involved in signal transduction from various environmental treatments and could also respond to the ratio between nitrogen and carbohydrates (Lea *et al*., 2007*b*). In *Lotus*, *LjPAP* and *LjTT2* were reported to enhance expression of enzymes involved in anthocyanidin and tannin biosynthesis (Yoshida *et al.*, 2010). Among other MYB transcription factors that could be related to the secondary metabolism, AtMYB4, AtMYB15, AtMYB20 and AtMYB96 orthologs were strongly over-expressed in *Ljgln2-2* mutant on air (Perez-Delgado *et al*., 2013). In WT plants, elicitation with reduced glutathione, which induced isoflavonoid production, also enhanced MYB4, MYB15 and MYB20 transcription factors, whereas repressed MYB96 (Shelton *et al.,* 2012), but further data about their possible functions in biosynthesis of secondary metabolites in *Lotus* are still missing. In *Arabidopsis*, MYB4 seems to repress flavonol biosynthesis (Zhao *et al.*, 2007), MYB15, MYB20 and MYB96 are involved in ABA signalling (Cui *et al*., 2013; Ding *et al.*, 2009). MYB96 interacted with PAP1 and PAP2 and resulted in enhanced anthocyanin biosynthesis and accumulation (Seo and Park, 2010).

Considering that several of the accumulated secondary metabolites in the *Ljgln2-2* mutant have antioxidant capacities, it is quite likely that most of the changes observed may be related to oxidative stress and/or signalling. In

addition, it is important to note that some compounds (mainly isoflavonoids) are pathogen-defence chemicals; others may be involved in nodulation initiation or in modulation of auxin transport (Agati *et al*. 2013; Besseau *et al*., 2007; Ferrer *et al*., 2008). Therefore, several other physiological processes in photorespiratory mutants may be also affected. The multiplicity of the functional roles of phenolic compounds and their interaction with plant metabolism and development of leaves is still plenty of open questions and offers an interesting matter for further investigation.

Asparagine breakdown in *L. japonicus*: the role of asparaginases

Many asparaginase enzymes have been described in the literature, but plant types have been less well studied than those from bacteria. Although different groups of bacterial asparaginases have been reported (Borek and Jaskolski, 2001), only aspartylglucosaminidases (EC 3.5.1.26) show some sequence similarity to the plant asparaginases. Plant asparaginases belong to the superfamily of N-terminal nucleophilic hydrolases (Ntn-hydrolases). The family of Ntn-hydrolases includes enzymes such as aspartylglucosaminidases (Guo *et al*., 1998; Oinonen *et al.*, 1995), penicillin acylases (Duggleby *et al.*, 1995; Kim *et al*., 2000; Suresh *et al.*, 1999), taspase1 (Khan *et al*., 2005) and plant-type asparaginases (Borek *et al*., 2004; Michalska *et al*., 2005; Michalska and Jaskolski, 2006; Michalska *et al*., 2006; Michalska *et al*., 2008). In Ntn enzymes, a cleavage of the precursor polypeptide chain is required in order to generate a catalytic residue at the newly formed N terminus, which can be a threonine, serine, or cysteine. In the case of asparaginases, two different subunits, α and β, are generated from a single autoproteolytic cleavage of a single precursor protein. Consequently, plant asparaginases are heterotetramers with a native molecular weight around 75 kDa (Credali *et al.*, 2011). They are dimers of heterodimers $(\alpha\beta)_2$ where each heterodimer is composed by one subunit α and one β (Fig. 6).

Among plant asparaginases, two different sub-groups can be distinguished based on the requirement of potassium for the enzyme activity. The presence of K^+-dependent or K^+-independent asparaginases in plants is well established (Bruneau *et al*., 2006; Cañas *et al.*, 2007; Sodek *et al.*, 1980). However, the exact meaning of the presence of potassium dependent and independent isoenzymes is poorly understood (Lea *et al.*, 2007*a*), in spite of the fact that potassium is the most abundant inorganic cation in plants, representing up to the 10% of a plant's dry weight (Leigh and Win Jones, 1984). Despite their separate classification, the

two groups of plant asparaginases share significant levels (about 60%) of sequence similarity.

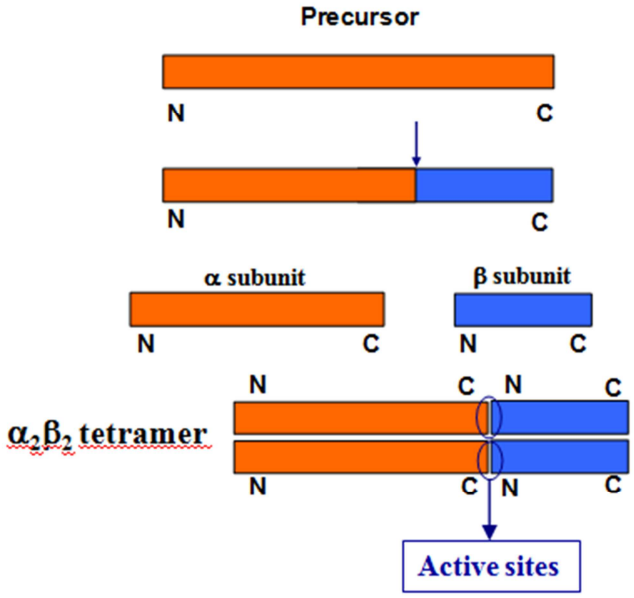

Fig. 6. Autoproteolytic processing of plant asparaginases to obtain the native enzyme conformation.

Structure and function of K^+-dependent and K^+-independent asparaginases from L. japonicus

The catalytic properties of a K^+-dependent (LjNSE1) and a K^+-independent (LjNSE2) asparaginase isoforms from *L. japonicus* were recently studied (Credali *et al.*, 2011). Recombinant LjNSE1 showed a K_m for asparagine of about 7.20 ± 1.67 mM in the presence of 50 mM K^+, and 17.90 ± 0.45 mM in its absence. On the other hand, LjNSE2 showed a K_m for asparagine around 30 mM, which did not change significantly by the presence of 50 mM K^+ in the assay mixture (29.2 ± 2.2 mM in the presence of 50 mM K^+ and 33.3 ± 6.7 mM in its absence) (Credali *et al.*, 2011). Therefore, it was shown that the presence of K^+ increased the affinity for asparagine by 3 times in the case of LjNSE1 enzyme, but not in the LjNSE2 isoform. In addition, it was also established that the level of LjNSE1 enzymatic activity and thermal stability were increased by 9- to 10-fold in the presence of K^+. Maximum LjNSE1 activity was found at 5-50 mM K^+, with a K_m for K^+ of 0.25 mM. Na^+ and Rb^+ can, to some extent, substitute for K^+ on the activating effect of LjNSE1 and were more efficient activators than Cs^+ and Li^+. The stimulating effect of the monovalent cation was correlated with ion's atomic radius. All these results clearly indicate that K^+ plays an important role in both asparaginase catalytic activity and stability of the LjNSE1 enzyme but not for LjNSE2 (Credali *et al.*, 2011).

The existence of an asparaginase isoform with a higher enzyme activity and higher affinity for asparagine must be of crucial importance for plants that use asparagine as a N transport compound, such as *L. japonicus*, and suggests that LjNSE1 must be the main enzyme responsible for the utilization of asparagine in this plant, in accordance with the highest level of expression of this particular isoform in sink tissues of the plant (Credali *et al.*, 2011).

The high K_m value for asparagine observed for LjNSE2 (around 30 mM), suggests that this particular isoenzyme may have a physiological role unrelated to the hydrolysis of asparagine *in vivo*, particularly because the asparagine concentration in xylem sap from *L. japonicus* is in the range of 6-17 mM (Waterhouse *et al.*, 1996). However, LjNSE2 enzyme shows an efficient β-aspartyl-hydrolase activity (K_m = 0.54 ± 0.02 mM for β-asp-his). This was not the case for the K^+-dependent isoform LjNSE1. The catalytic efficiency of the LjNSE2 isoform is 12-fold higher with β-aspartyl-histidine hydrolase than asparaginase activity. As various isoaspartyl peptides are known to arise from proteolytic degradation of post-translationally altered proteins containing isoaspartyl residues, plant-type asparaginases may not only function in asparagine catabolism but also as detoxifying enzymes (Bruneau *et al.*, 2006). According to this proposal, K^+-independent LjNSE2 is probably a detoxifying enzyme in *L. japonicus*. However, the K^+-independent (ASPGA1) and K^+-dependent (ASPGB1) asparaginases from *Arabidopsis thaliana* were found to be catalytically active with both L-asparagine and β-Asp-His as substrates, having ASPGB1 a 45-fold higher specific activity with asparagine as substrate than ASPGA1 (Gabriel *et al.*, 2012). These results establish some differences with regard to asparaginase behaviour in *L. japonicus* compared to *A. thaliana*, in spite of the presence in both species of K^+-dependent and K^+-independent isoforms (Bruneau *et al.*, 2006; Credali *et al.*, 2011).

Mass spectrometric analysis of purified asparaginase protein preparations revealed a molecular mass of 20.90 ± 0.15 kDa and 17.11 ± 0.15 kDa for α and β subunits of recombinant LjNSE1 respectively (including an His-tag tail), and 20.18 ± 0.15 kDa and 17.33 ± 0.15 for the same subunits in LjNSE2. These results are compatible with the existence of a single protein cleavage site placed at the peptide bond next to the expected highly conserved Thr residue previously reported to be catalytically active (Thr196 for LjNSE1 and Thr187 for LjNSE2), similarly to other plant asparaginases described (Credali *et al.*, 2011).

So far *Lupinus luteus* K^+-independent asparaginase is the only plant asparaginase that has been crystallized (Michalska *et al.*, 2006). Using *L. luteus* crystal structure as a template, models of *L. japonicus* K^+-dependent (LjNSE1) and K^+-independent (LjNSE2) asparaginases have been created and the two proteins belonging to the different plant asparaginase subfamilies have been compared (Credali *et al.*, 2011). As expected, the (αβ) heterodimers have the classical αββα sandwich Ntn-hydrolase fold with a protein core composed of two open β-sheets surrounded on each side by an α-helix layer. The (αβ)–(αβ) interface is additionally cemented by a number of hydrophobic interactions, with contribution from all four subunits. The hydrophobic side-chains are scattered throughout the entire interface. The two β-sheets at the core of the LjNSE1 and LjNSE2 proteins were highly similar. In contrast, two low identity regions were found. The first corresponds to residues 16-35 (loop 1 in α-helix 1 in α-subunit) whose structure is conserved but with a different net charge. The second low identity stretch spans from residues 158 to 195 in LjNSE1, which correspond to the variable loop at the C-end in the α-subunit.

Electrostatic surface potentials (ESP) of the two proteins were similar but LjNSE1 showed negative patches that were absent in LjNSE2. The most significant one was located at the rim of the active site crevice and is formed by Glu248, Asp285 and Glu286 (Fig. 7), that are replaced in LjNSE2 by Lys239, Pro276 and Lys277 respectively. These three amino acid residues were found to be crucial for K^+ interactions with LjNSE1 as determined by site-directed mutagenesis (Credali *et al.*, 2011). It is frequent that metal binding sites in proteins are formed by small loops in the polypeptide that provide a set of 3-4 ligands and thus define the site (Williams, 1998). K^+-dependent LjNSE1 asparaginase appears to be another of such examples where the above mentioned ligands must contribute to the coordination of K^+. Considered that 6 ligands in octahedral configuration is the most commonly observed geometry for the monovalent cation coordination (Page and Di Cera, 2006), it is likely that at least one and possibly two water molecules could be involved in the completion of the K^+ coordination in the LjNSE1 from *L. japonicus* and may be in other K^+-dependent plant asparaginases. Other molecular dynamics and docking studies further confirmed the crucial relevance of K^+ for the proper orientation of asparagine substrate within the enzyme molecule and other features of LjNSE1 protein structure (Credali *et al.*, 2011). Recently, Gabriel *et al.* (2012) have suggested a role also for the asparaginase variable loop in the determination of substrate preference in the plant asparaginases. Homology modelling identified in the K^+-dependent asparaginase from *A. thaliana* (ASPGB1) the Phe162 residue, preceding the variable loop, whose side chain is located in proximity to

the β-carboxylate group of the product aspartate, and Gly246, a residue participating in an oxyanion hole, which stabilizes a negative charge forming on the side chain oxygen of asparagine during catalysis.

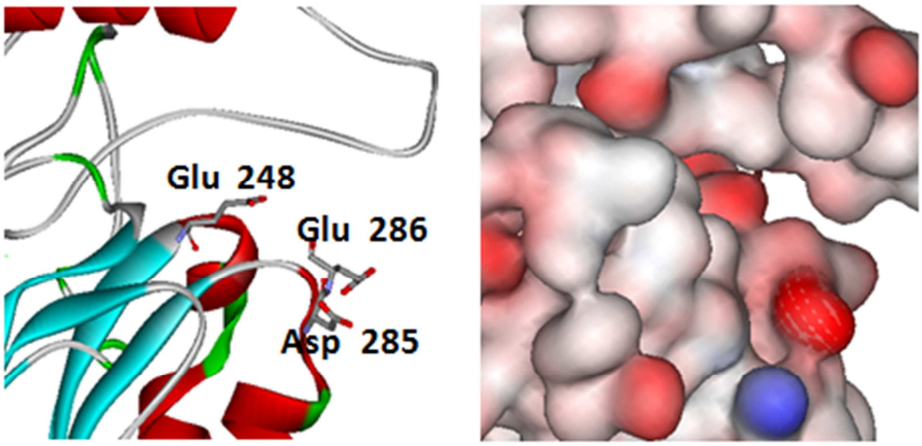

Fig. 7. Negative patch region within the LjNSE1 asparaginase molecule that binds K^+.

Concerning the potassium effect, enzymes can be classified into those that rely on high and relatively stable concentrations of K^+ in certain cellular compartments and those that depend on K^+ mobility between different compartments, cells, or tissues. Our results indicate that K^+-dependent asparaginase will belong to the first class function that includes enzyme activation, stabilization of protein synthesis, and neutralization of protein negative charges (Marschner, 1995).

Use of TILLING mutants for the study of K^+-dependent LjNSE1 *asparaginase functionality*

L. japonicus contains three asparaginase isoforms, named respectively LjNSE1, LjNSE2 and LjNSE3. According to their protein sequence data, two of these isoforms look to be K^+-dependent (LjNSE1 and LjNSE3), and the other one is K^+-independent (LjNSE2). A differential expression of these isoforms was observed in different organs of this plant (Credali, 2011). K^+-dependent LjNSE1 is by far the most highly expressed in many tissues of the plant. The reverse genetics TILLING tool (Targeted Induced Local Lesions IN Genomes, Perry *et al*., 2003) enabled to identify several mutants affected in the *LjNSE1* gene from *L. japonicus* (Credali *et al.*, 2013). Four different mutants were found and characterized, two of which (L230F and G206R) were affected in the structure and function of the asparaginase molecule and caused asparagine accumulation. The Leu230 residue is located at the interface of the two αβ dimers, and replacement of Leu230 by the aromatic residue phenylalanine results in several

steric constraints. On the other hand, Gly206 residue is positioned in the turn of two β strands and replacement of the small residue Gly206 by arginine affects the electrostatic environment within the protein and alters the structure of asparaginase. Analysis of these deleterious mutations enabled to show that LjNSE1 is not determinant for the nodulation of *L. japonicus* plants. Several studies were carried out to test if the nodulation performance was affected in the mutant plants. Although the *L. japonicus* NSE1 deficient mutants showed a much lower number and fresh weight of nodules compared to the WT, however, the specific ratio between fresh weight of nodules and fresh weight of roots was not significantly altered, indicating that the lower nodulation performance of the mutants must be due to their smaller size, but not to an essential requirement of LjNSE1 for nodulation (Credali *et al*., 2013). In contrast, LjNSE1 protein seems to be crucially relevant for plant growth and seed production. In fact, the size of NSE1 deficient mutant plants was much smaller than the WT at different times of growth. Stem length and leaf fresh weight were about 60-90% reduced at 35 days after sowing. A 70-80% decrease in root weight was also observed. Other growth parameters (stem number, trifoliate leaf number and root length) were also lower in the mutant plants affected in NSE1. Regarding to the effects produced by LjNSE1 deficiency on seed production, it was found that the mutant pods were shorter and contained a high proportion (up to 45%) of abnormal seeds, which were significantly smaller and had a shape completely different to that of the normal *L. japonicus* seeds. Aborted seed formation was also detected in the NSE1 deficient mutant plants. In addition, thin sections of normal seeds from WT and mutants resulted fairly similar, but, in contrast, histological sections of abnormal seeds indicated that no embryos were visible. Further work was carried out to investigate the effects of asparaginase deficiency on seed storage protein accumulation. It is known that mature WT *L. japonicus* seeds contain approximately 40% protein mainly in the form of the storage globulins (legumin and convicilin) (Dam *et al.*, 2009). It could be demonstrated that the NSE1 mutant seeds had lower storage globulins content than the WT seeds. However, the ratio between legumin (LLPs) and convicilin (LCPs) storage proteins was not significantly altered (Credali *et al.*, 2013).

All these results on the analysis of *Ljnse1* mutants point out a key difference between legumes such *L. japonicus* and other plants such as *A. thaliana*, where it was recently shown that asparaginase mutants develop normally, but exhibited enhanced root inhibition by exogenous asparagine (Ivanov *et al*., 2011). This weak phenotype in Arabidopsis was attributed to the fact that asparagine only accounts for approximately 5% of total amino acids in

the phloem sap of Brassicaceae, whereas it accounts for 86% of nitrogen translocated in *L. japonicus*.

Asparagine and photorespiration

It has been well known for a long time that asparagine can be used as nitrogen donor for transaminase reactions associated with the nitrogen photorespiratory cycle (Givan *et al.*, 1988; Joy, 1988). Asparagine aminotransferase was characterized from pea leaves and appeared to be the same protein as the peroxisomal photorespiratory enzyme serine-glyoxylate aminotransferases (SGAT) (EC 2.6.1.45), based on its substrate preference and subcellular localization (Ireland and Joy, 1983*a* and 1983*b*). Indeed, tobacco and barley mutants lacking SGAT were devoid of asparagine aminotransferase activity (Havir and McHale, 1988; Murray *et al.*, 1987). In addition, it has been recently demonstrated that a recombinant SGAT enzyme has a 5-20 fold higher V_{max} and a higher catalytic efficiency for asparagine compared to serine or alanine (Zhang *et a*l., 2013). All these results encourage further work to be carried out in order to determine the exact significance of asparagine transamination in photorespiration. The availability now of *Ljnse1* mutants from *L. japonicus* that accumulate higher amounts of asparagine, makes it very interesting to determine if there is some interconnection between asparaginase deficiency and SGAT or other photorespiration-related processes in *L. japonicus* plants.

CONCLUDING REMARKS

In this review we have summarized the recent progress made on the characterization of two of the major sources of endogenous ammonium in plants, such as photorespiration and the asparaginase reaction. We used for this purpose the model legume *L. japonicus*, which is the focus for modern genome sequencing and functional genomics programs (Márquez, 2005, Udvardi *et al.*, 2005, and references therein). The results described in this paper highlight the crucial importance of ammonium reassimilation in the legume plants and the usefulness of the mutants analyzed for future studies on plant nitrogen nutrition.

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Publicación 8.

Genes for ammonium assimilation.

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ABSTRACT

Ammonium resulting from primary nitrate reduction, dinitrogen fixation or nitrogen remobilization has to be efficiently assimilated. In this chapter we describe the main enzymes and genes responsible for ammonium assimilation in *L. japonicus* plants. We summarize the nomenclature and codes available in Kazusa 2.5 for the main genes involved in the ammonium assimilatory process, as well as the levels of expression found by qRT-PCR for these genes in different tissues of the plant.

1. INTRODUCTION

In the model legume *Lotus japonicus* different forms of inorganic nitrogen (NO_3^-or NH_4^+) can be taken up by the plants, depending on nitrogen availability (Márquez et al. 2005; Orea et al. 2005). The utilization of NO_3^- requires its reduction to NH_4^+ produced by the consecutive action of nitrate reductase and nitrite reductase enzymes, prior to ammonium assimilation. Previous works have characterized the nitrate and nitrite reduction systems in *L. japonicus* (Harrison et al. 2004; Márquez et al. 2005; Orea et al. 2001; Pajuelo et al. 2002; Prosser et al. 2006). On the other hand, *L. japonicus* as a legume plant, has also the chance to establish symbiosis with *Mesorhizobium loti* bacteria in order to use atmospheric N_2, which is reduced to NH_4^+ in the nodules by the action of bacterial nitrogenase (see other chapters of this book). Consequently, the process of primary ammonium assimilation, either derived from nitrate reduction or dinitrogen fixation, is of crucial importance in *L. japonicus* plants. This is the case also for other processes that produce an internal release of ammonium in *L. japonicus* plants, such as photorespiration, phenylpropanoid biosynthesis or amino acid catabolism, called in general terms as secondary ammonium assimilation (Betti et al. 2012; Márquez et al. 2005).

In the following sections we will describe first the main enzymes in charge of ammonium assimilation, basically associated with glutamine/glutamate and asparagine metabolisms (section 2). This is followed by the description (section 3) of the different genes from *L. japonicus* which encode for the aforementioned enzymes, their nomenclature and the codes available for them in Kazusa 2.5 database as well as the corresponding ortholog genes in *A. thaliana*. We also comparatively analyze the levels of expression determined by qRT-PCR that we have obtained for all these genes in leaves, roots and nodules of *L. japonicus* plants growing under N-sufficient conditions. All these results are summarized also in Table 1.

Gene Name	Code in Kazusa 2.5	Levels of expression			Ortholog genes in *A. thaliana*
		Leaves	Roots	Nodules	
LjGln1.1	*chr2.CM0312.1480.r2.m*	H	H	H	*At5g37600 (AtGln1.1)*
LjGln1.2	*chr6.CM0014.300.r2.m*	VH	VH	VH	*At1g66200 (AtGln1.2)*
LjGln1.3	*LjSGA_030247.1*	L	VL	VL	*At3g17820 (AtGln1.3)*
LjGln1.4	*LjSGA_058827.1*	L	ND	ND	*At5g16570 (AtGln1.4)*
LjGln1.5	*LjSGA_019428.1*	VL	ND	ND	*At1g48470 (AtGln1.5)*
LjGln2	*chr6.CM0139.890.r2.m*	VH	VH	VH	*At5g35630 (AtGln2)*
LjGlu1	*chr1.CM0009.170.r2.d*	VH	H	H	*At5g04140 (AtGlu1)* *At2g41220 (At Glu2)*
LjGlt1	*LjSGA_035611.1*	VL	L	L	*At5g53460 (AtGlt1)*
LjGlt2	*LjSGA_037992.1*	H	H	H	
LjGdh1	*chr1.CM0104.2530.r2.m*	H	VH	L	*At5g18170 (AtGdh1)*
LjGdh2	*chr4.CM2142.210.r2.a*	VL	L	VL	*At5g07440 (AtGdh2)*
LjGdh3	*chr2.CM0021.1320.r2.m*	ND	VL	ND	*At3g03910 (AtGdh3)*
LjGdh4	*chr3.CM1488.210.r2.d* *chr3.CM1488.260.r2.d* *chr3.CM1488.250.r2.d* *chr3.CM1488.230.r2.d*	H	H	H	*At1g51720 (AtGdh4)*
LjAsn1	*chr5.CM0071.330.r2.d*	VH	VH	H	*At3g47340 (AtAsn1)*
LjAsn2	*LjT47C13.80.r2.d*	VL	VH	VH	*At5g65010 (AtAsn2)*
LjAsn3	*LjT09J04.190.r2.d*	L	L	L	*At5g10240 (AtAsn3)*
LjNse1	*chr5.CM0096.20.r2.m*	VH	H	L	*At3g16150 (AtAspGB1)*
LjNse2	*chr4.CM0087.1740.r2.m*	L	H	H	*At5g08100 (AtAspGA1)*
LjNse3	*LjSGA_021574.1*	H	L	VL	*At3g16150 (AtAspGB1)*

Level of expression (relative units)	Level of expression	Symbol
0 - 0.001	Undetectable	ND
0.001 - 0.01	Very Low	VL
0.01 - 0.1	Low	L
0.1 - 1	High	H
1 - 10	Very High	VH

Table 1. Genes for ammonium assimilation in *L. japonicus*

2. Enzymes involved in ammonium assimilation

2.1. The GS-GOGAT cycle

Glutamine is the first organonitrogen compound that is synthesized in the plants as a result of both primary and secondary ammonium assimilation, by means of the glutamine synthetase (GS) / glutamate synthase (GOGAT) cycle. Glutamine synthetase (EC 6.3.1.2) catalyses the biosynthesis of L-glutamine from L-glutamate, ATP and NH_4^+ according to the following reaction, that requires also Mg^{2+} cations as cofactors:

$$\text{L-glutamate} + \text{ATP} + NH_4^+ \rightarrow \text{L-glutamine} + \text{ADP} + P_i$$

Subsequently, the glutamate synthase enzyme (EC 1.4.7.1 or EC 1.4.1.14) catalyses the transfer of the amide group of glutamine into 2-oxoglutarate, yielding two molecules of glutamate, a reaction requiring also two electrons coming either from reduced ferredoxin (Fd-GOGAT) or pyridine nucleotides (NADH-GOGAT), as follows:

$$\text{L-glutamine} + \text{2-oxoglutarate} + 2\,e^- + 2\,H^+ \xrightarrow{GOGAT} 2\,\text{L-glutamate}$$

The global balance of the consecutive action of these two enzymes forms the GS-GOGAT cycle by which one of the two molecules of glutamate formed by the GOGAT would be used by the reaction of GS. Consequently the GS-GOGAT pathway results in the net formation of one molecule of L-glutamate at the expenses of one molecule of 2-oxoglutarate, one molecule of NH_4^+ and one molecule of ATP as follows:

$$\text{2-oxoglutarate} + NH_4^+ + 2\,e^- + 2\,H^+ + \text{ATP} \xrightarrow{GS\text{-}GOGAT} \text{L-glutamate} + \text{ADP} + P_i$$

2.2. Glutamate dehydrogenase

In addition to GS and GOGAT, which catalyse irreversible reactions, a third enzyme, glutamate dehydrogenase (GDH; EC 1.4.1.2/4) catalyses a reversible amination/deamination reaction, which could lead to either the synthesis or the catabolism of glutamate, according to the following equation:

$$\text{2-oxoglutarate} + NH_4^+ + \text{NAD(P)H} + H^+ \overset{GDH}{\longleftrightarrow} \text{L-glutamate} + \text{NAD(P)}^+$$

The role of GDH in glutamate catabolism is quite well established. Instead, the possible anabolic role of GDH for the assimilation of ammonium has been the subject of continuous controversy because most lines of evidence support that glutamate biosynthesis takes place through the GS-GOGAT pathway although it has been also reported a role for GDH under different plant stress situations. It has been proposed that GDH has an important meaning in terms of metabolic signaling in relation to partitioning of C and N assimilates being likely that GDH contributes to the control of the homeostasis of leaf glutamate, a process of crucial importance (Fontaine et al. 2012).

2.3. Asparagine metabolism: asparagine synthetase and asparaginase

In most temperate legumes, it is proposed that asparagine, rather than glutamine, is the principal molecule used to transport reduced nitrogen within the plant, in contrast to many other plant species (Credali et al. 2013). This is the case for *L. japonicus* where it has been shown that asparagine can account for

almost 90 % of the nitrogen transported from root to shoot (Waterhouse et al. 1996).

Asparagine synthetase (AS, EC 6.3.5.4) is the main enzyme in charge of asparagine biosynthesis in plants. This enzyme catalyzes the transfer of the amide group from glutamine to aspartate in an ATP-dependent reaction:

$$\text{L-glutamine + L-aspartate +ATP} \xrightarrow{AS} \text{L-glutamate + L-asparagine + AMP + } PP_i$$

It has been also proposed that the enzyme can use high concentrations of ammonia directly as substrate, but this is not clearly demonstrated (Lea et al. 2007).

Considering that asparagine is a nitrogen transport compound in *Lotus*, asparagine breakdown is also a process of crucial importance for this plant (Credali et al. 2013). Asparaginase (NSE, EC 3.5.1.1) catalyses the hydrolysis of asparagine to yield aspartate and ammonia as follows:

$$\text{Asparagine} + H_2O \xrightarrow{NSE} \text{Aspartate} + NH_4^+$$

The ammonia released by the asparaginase reaction has to be subsequently reassimilated by GS (Lea et al. 2007)

3. Genes for ammonium assimilation

3.1. Glutamine synthetase (GS)

As in other plant species, a small multigene family is responsible for GS enzymatic activity in *L. japonicus*. Five gene sequences encoding for cytosolic GS (also called *GS1* or *Gln1*) and another one for plastidic GS (also called *GS2* or *Gln2*) were found in the available databases. Two of the cytosolic genes are expressed at high (*LjGln1.1)* or very high *(LjGln1.2)* levels in leaves, roots and nodules from this plant (Table 1). The level of expression of the *LjGln1.2* gene is about 3-fold higher in nodules than in roots or leaves, while for the case of *LjGln1.1* gene, the level of expression is lower in nodules and leaves than in roots (results not shown). The third gene (*LjGln1.3*) is expressed at low level in leaves and at very low levels in roots and nodules. A fourth and a fifth cytosolic gene (*LjGln1.4* and *LjGln1.5)* are only expressed at low (*LjGln1.4)* or very low (*LjGln1.5)* levels in leaves. Cytosolic GS polypeptides are the most abundant both in roots and nodules of *L. japonicus* plants (García-Calderón et al. 2012; Orea et al. 2002). Previous studies in *L. japonicus* had indicated that a high level of GS activity in the root is negatively correlated with above-ground biomass (Limami et al. 1999). On the other hand, lowering GS activity in nodules results

in an increase in fresh weight in nodules, roots and shoots (Harrison et al. 2003). This change in biomass could be explained by more efficient ammonium assimilation in the nodules of transformed plants, as indicated by a large increase in amino acids (mostly asparagine) with a concomitant decrease in carbohydrate content (Harrison et al. 2000). Other results established that constitutive overexpression of GS1 in *Lotus* produce higher amino acid levels and soluble protein concentration, higher chlorophyll content and a higher biomass accumulation in the transgenic plants (Ortega et al. 2004) while overexpression in shoots may accelerate plant development, leading to early senescence and premature flowering when plants are grown on an ammonium-rich medium (Vincent et al. 1997). In addition, it was also described that overexpression of GS1 in reproductive organs critically affects their development and might be a reason for sterility of *L. japonicus* plants (Suárez et al. 2003). In other plant species 2-5 functional *Gln1* genes have been generally reported. Different versions of these genes have been found to be associated with different roles in primary ammonium assimilation and other forms of N recycling in response to nitrogen availability in the external medium, plant nitrogen status, light/dark conditions or abiotic/biotic stressors (Bernard and Habash 2009). Cytosolic GS assimilates ammonium from the three major types of nitrogen-fixing symbiotic association involving plants and either *Rhizobium*, actinomycetes such as *Frankia*, or cyanobacteria. In some species a specific cytosolic GS isoenzyme is induced in nitrogen-fixing root nodules, whereas, in others, cytosolic GS already present is involved (Bernard and Habash 2009).

Regarding to *Gln2*, a single gene has been detected in *L. japonicus*, which has very high levels of expression either in leaves, roots or nodules (Table 1). Nevertheless, the levels of transcript detected in leaves were found to be about 5-fold higher than those present in roots or nodules from this plant (results not shown). In most plant species plastidic GS (GS2) is exclusively or very predominantly expressed in green tissues. However, the presence of GS2 was clearly demonstrated in non-photosynthetic tissues of temperate legumes (García-Calderón et al. 2012). In *Medicago truncatula*, a second *Gln2* gene was recently shown to be exclusively expressed in developing seeds (Seabra et al. 2010). The first *gln2* mutants available from legume plants were isolated from *L. japonicus* belonging to the class of photorespiratory mutants previously described in other plant species (Márquez et al. 2005; Orea et al. 2002;). The *Ljgln2* mutants were further characterised at the molecular level showing to be affected by single point mutations within the structural part of the *LjGln2* gene, which lead to amino acid replacements that abolish GS2 enzymatic activity completely (Betti et al. 2006). These mutants were used to analyze how photorespiratory metabolism affects nodule function in *L. japonicus* plants

(García-Calderón et al. 2012). The results obtained indicated that in this plant photorespiration, and, particularly, GS2 deficiency result in profound limitations in carbon metabolism that affect the nodulation process and nitrogen fixation. An anticipated senescence phenotype linked to an important reduction in starch and sucrose levels was observed (García-Calderón et al. 2012). On a separate work, a single *LjGln2* locus encoding for GS2 was mapped together with other symbiotic loci (Sandal et al. 2006). More recently, GS2 was also involved in drought stress, nitrogen nutrition and photorespiratory metabolism transcriptomic responses in *L. japonicus* plants (Betti et al. 2012; Díaz et al. 2010; Pérez-Delgado et al. 2013).

3.2. Glutamate synthase (GOGAT)

One *LjGlu1* gene encoding for Fd-GOGAT and two different genes for NADH-GOGAT (*LjGlt1* and *LjGlt2*) were identified in Kazusa database. Expression levels of *LjGlu1* were very high in photosynthetic tissues but also were high in roots and nodules, as it also happened with *LjGlt2* whose expression levels were also high in the three types of tissues. However, this was not the case for *LjGlt1* gene which was poorly expressed in roots, nodules and leaves (Table 1). Early studies indicated that NADH-GOGAT appeared to play a major role in legume root nodules, in which the activity increases dramatically following the onset of nitrogen fixation. Two different isoforms of NADH-GOGAT have been described in other plant species, one of them clearly associated to effective nodules (Ireland and Lea 1999, and references therein). Measurements of mRNA levels and promoter-GUS fusions of the NADH-GOGAT genes in alfalfa and *Lotus* have shown the tight relationship of the regulated expression of NADH-GOGAT to the nodulation process in legumes (Vance et al. 1995).

3.3. Glutamate dehydrogenase (GDH)

Three different genes encoding for the NAD^+-dependent GDH were identified in *L. japonicus* (Table 1). One of them (*LjGdh1*) showed high or very high levels of expression in leaves and roots respectively and low level in nodules. The other two genes (*LjGdh2* and *LjGdh3*) were poorly expressed in these tissues. The majority of recent studies performed on NAD^+-GDH in higher plants have been focused on deciphering the role of the α- and β-subunits in the formation of seven isoenzymes which are encoded by two distinct nuclear genes, *Gdh2* and *Gdh1*, respectively. More recently it has been found that in *Arabidopsis* there is a third gene *(AtGdh3)* encoding a putative NAD^+-GDH that is actively transcribed and perhaps regulated by cytokinin. Similarly, in rice three genes encoding NAD^+-GDH, were reported, and also in soybean, although it seems that the physiological functions of the GDH isoenzymes is a complex

issue and may vary from one species to another (Fontaine et al. 2012, and references therein).

A $NADP^+$-dependent form of GDH also exists, which appears to be localized in the chloroplast (in contrast to NAD^+-GDH which appears to be localized in mitochondria). However, the role of $NADP^+$-GDH is not clear. Consequently, a fourth expressed gene (*AtGdh4*) encoding a putative $NADP^+$-GDH has been identified in *Arabidopsis* and rice, being 50 % longer than the NAD^+-GDH (Fontaine et al. 2012, and references therein). This is the case also in *L. japonicus*, where a *LjGdh4* gene was also identified in Kazusa, which is associated to 4 different code names (Table 1). There is a high level of expression of the *LjGdh4* gene in leaves, roots and nodules from this plant.

3.4. Asparagine synthetase (AS)

Three genes for asparagine synthetase (*LjAsn1*, *Lj*Asn2 and *LjAsn3)* have been identified in *L. japonicus* (also called respectively *LjAS1*, *LjAS2*, *LjAS3*). *LjAsn1* is highly or very highly expressed in mature leaves, roots and nodules. *LjAsn2* is very highly expressed in roots and nodules and barely detectable in leaves.The third gene (*LjAsn3*) is poorly expressed in the three types of tissues (Table 1). The molecular cloning and characterisation of AS from *L. japonicus* in relation to the dynamics of asparagine biosynthesis in N-sufficient conditions, has been reported previously (Waterhouse et al. 1996). Three genes encoding AS have been identified in other plant species, such as *A. thaliana* which appear to be regulated in different manners (Lea et al. 2007). Although there is considerable variation between plants in the exact mechanisms involved in the regulation of the expression of AS, there is an overall consensus: the expression of one gene (often that which is most highly expressed) is induced by a reduction in soluble carbohydrate supply and in some cases darkness, while a second gene is more widely expressed but may be stimulated by carbohydrate and light. An increased supply of reduced nitrogen, either as ammonium or amino acids, induces expression of AS genes (Lea et al. 2007). Analysis of the amino acid sequences of plant ASs shows that the proteins contain glutamine, aspartate and AMP binding sites and are related to the *E. coli* asparagine synthetase ASB glutamine-dependent enzymes (Lea et al. 2007).

3.5. Asparaginase (NSE)

Three different *Nse* genes encoding for asparaginase have been identified in *L. japonicus*, which showed a different pattern of expression among leaves, roots and nodules of the plants (Table 1). The *LjNse1* gene is by far the most highly expressed one, particularly in leaves. Two of the genes present in *L.*

japonicus, named *LjNse1* and *LjNse3*, encode for different K^+-dependent versions of the asparaginase enzyme, while a third one, named *LjNse2* corresponds to a K^+-independent isoform. All these enzymes have a $\alpha_2\beta_2$ tetrameric quaternary structure, where the α and β subunits correspond respectively to the N-terminal (20 kDa) or C-terminal (17 kDa) domains arising from a single proteolytic event of each precursor which is encoded by the different *Nse* genes. Structural and kinetic studies revealed the crucial importance of K^+ for the higher enzymatic activity and stability as well as lower K_m for asparagine and proper orientation of asparagine substrate within the LjNSE1 enzyme molecule (Credali et al. 2011). It was proposed that LjNSE1 must be the main enzyme responsible for the utilization of asparagine in *L. japonicus* plants, while the K^+-independent isoform LjNSE2 is probably a detoxifying enzyme in charge of the release of isoaspartyl peptides arising from proteolytic degradation of post-tranlationally altered proteins. TILLING mutants affected in *LjNSE1* asparaginase isoform were recently used to demonstrate by reverse genetics the importance of this particular isoform in plant growth and seed production. In fact, the level of both legumin and convicilin seed storage proteins were affected in the mutants (Credali et al. 2013). Nevertheless *nse1* mutants indicated that there was no apparent involvement of *NSE1* protein in nodulation. Interestingly, these results illustrate on a key difference between *L. japonicus* and *Arabidopsis* where asparaginase activity seems to be dispensable; in fact *aspG* insertional mutants from *Arabidopsis* lacking of one or both K^+-dependent *(AspGB1)* and K^+-independent (*AspGA1)* asparaginases develop normally (Ivanov et al. 2011). This differential behaviour among *Lotus* and *Arabidopsis* regarding to asparaginases was attributed to the fact that asparagine only accounts for approximately 5 % of total amino acids in the phloem sap of Brassicaceae, whereas it accounts for almost 90 % of nitrogen translocated in *L. japonicus* (Waterhouse et al. 1996), thus highlighting the importance of asparagine for nitrogen remobilization in *L. japonicus* plants (Credali et al. 2013).

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Publicación 9.

A *Lotus japonicus* mutant deficient in nitrate uptake is also affected in the nitrate response to nodulation.

Pal'ove-Balang P, García-Calderón M, Pérez-Delgado CM*, Pávlovin J, Betti M, Márquez AJ (2014) *Plant Biology* (en prensa). *co-primera autora

ABSTRACT

A chlorate-resistant mutant (*Ljclo1*) of the model legume *Lotus japonicus* was identified that showed normal levels of nitrate reductase enzyme activity but it had a decreased uptake of nitrate, as determined by nitrate depletion and electrophysiological measurements. The data obtained suggest that the mutant could be affected specifically in the low-affinity but not in the high-affinity nitrate transport systems, and also showed a decreased uptake of chlorate. Back-crosses of the mutant plant to the wild type indicated that it is affected in a single Mendelian recessive trait. Thus, the mutation produced in *Ljclo1* may be related to some of the low-affinity nitrate transporters or to a regulatory mechanism associated with nitrate/chlorate uptake. The size of the mutant plants and the chlorophyll content in the young leaves were significantly reduced compared to the wild type. In addition, it was also observed that nodulation performance of the mutant plants was similar to the wild type in the absence of any exogenous nitrate. However, the nodule: root biomass ratio in the mutant plants was considerably reduced in the presence of 1-2 mM nitrate. The levels of several transcripts for nitrate transport and assimilation related genes were determined for the wild type and mutant plants and shown to be slightly different. The results obtained suggest an interdependence between nitrate uptake, plant growth and nodulation in *Ljclo1* mutant plants.

INTRODUCTIÓN

The major source of inorganic nitrogen available to plants is a mixture of nitrate and ammonium, with nitrate being the predominant form in well-aerated soils as a consequence of bacterial nitrification. The nitrate present in the soil solution is then taken up by roots and assimilated within plant cells by three sequential steps: (i) Transport across the plasma membranes, using a variety of high-affinity or low-affinity transport systems; (ii) Reduction of nitrate to nitrite and nitrite to ammonium, through the consecutive action of nitrate reductase (NR) and nitrite reductase (NiR) enzymes; (iii) Assimilation of ammonium into organic nitrogen, yielding glutamine and glutamate as the primary organic nitrogen compounds that distribute nitrogen to all other N-containing metabolites and macromolecules. Glutamine synthetase (GS) and glutamate synthase (GOGAT) are the key enzymes responsible for primary ammonium assimilation in higher plants, both of ammonium derived from nitrate as well as ammonium taken up directly from the exterior. A set of isoforms of all these enzymes, having different patterns of expression in different tissues, is a distinctive peculiarity of higher plants (Márquez *et al.* 2005; Masclaux-Daubresse *et al.* 2010).

Nitrate transport and signalling is an active area of research in plants. An ample variety of nitrate transporters have been identified over the past few years. Two basic types of nitrate transport systems have been shown to coexist in plants and to act co-ordinately to take up nitrate from the soil solution and distribute it within the whole plant. The high-affinity transport system (HATS) displays Michaelis–Menten kinetics saturating at 0.2–0.5 mM nitrate. The low-affinity transport system (LATS) is observed at concentrations above 0.5 mM, and usually displays non-saturating uptake kinetics. Plants have multiple nitrate carriers with distinct kinetic properties and regulation. Two basic nitrate transporter gene families have been reported to be in charge of nitrate transport and were named *NRT1/PTR* (recently renamed as *NPF* as proposed by Leran *et al*. 2013) and *NRT2*. The *NRT2* family encodes transporters that contribute to HATS while the *NPF* family is basically responsible for LATS although particular members of this family have either high or dual (high and low) affinities. The recent work of Glass & Kotur (2013) has claimed that the dual affinity of AtNRT1.1 (AtNPF6.3) can be applied only to *X. laevis* oocyte system and not to the *in planta* studies. Other membrane bound transporters, such as chloride channel (CLC), have been also associated to nitrate transport in the plants. All these transporters or channels have provided new insights into the molecular mechanisms of nitrate uptake and allocation. Interestingly, several of these transporters also play versatile roles in nitrate sensing, plant development,

pathogen defense, and/or stress response (for reviews see Forde 2000; Fernández & Galván, 2008; Gojon *et al.* 2011; Miller *et al.* 2007; Orsel *et al.* 2002; Wang *et al.* 2012, and references therein).

In addition to nitrate and ammonium nutritions, several plant species, most notably legumes, are able also to develop symbiotic processes with bacteria in order to use atmospheric dinitrogen, which is reduced to ammonium in the nodules by the action of bacterial nitrogenase. Under N-limited conditions, legumes absorb mineral nitrogen and particularly nitrate to fulfil their nutritional demand before functional symbiotic nodules are differentiated. Small supplements of combined N, such as NO_3^-, NH_4^+, and urea, added at sowing may benefit symbiosis by increasing seedling growth rate and the number, size, and efficiency of the resulting nodules, whereas the addition of excessive amounts of combined N (nitrate or ammonium) depresses nodulation. However, little is known about how combined N sources interfere with the symbiotic program (Barbulova *et al.* 2007). It was recently shown that a variety of regulatory mechanisms are involved in the nitrogen-dependent modulation of the nodule organogenesis program in legume roots (Omrane & Chiurazzi 2009). Whether NO_3^- repressive effects are due to the ion itself, to products of its assimilation, or both, is not yet clearly demonstrated (Morere-LePaven *et al.* 2011).

The study of nitrate transporters in legumes has to date received little attention, probably because these species are able to adapt to N starvation by promoting symbiosis (Morere-LePaven *et al.* 2011). Previous works established the presence both in *Lotus japonicus* and soybean of anion transporters of the symbiosome membrane with transport preference for NO_3^- (Vincill *et al.* 2005). Recent work has established that the *NPF1.7* gene from *Medicago truncatula* encodes a member of the *NPF* transporter family which behaves as a high-affinity nitrate transporter with a second unknown function (Bagchi *et al.* 2012). The first functional characterization of a *M. truncatula* nitrate transporter, MtNPF6.8, was also reported recently (Morere-Le Paven *et al.* 2011). This transporter probably does not have a major role in overall root NO_3^- acquisition but may rather be involved in the plant response to NO_3^- starvation, because it was shown that, unlike members of the NPF superfamily characterized so far, the expression of *MtNPF6.8* in roots is stimulated locally by N starvation and repressed in medium supplied with NO_3^- (Morere-LePaven *et al.* 2011). However, the information concerning mutants affected in nitrate transport from legumes is very scanty.

Chlorate resistant mutants have been particularly useful for the isolation

and characterization of nitrate reductase or nitrate transport mutants in plants because of their inability to take up or reduce chlorate (nitrate analogue) to toxic chlorite (Navarro *et al.* 2005 and references therein). In this paper we report on the characterization of a chlorate-resistant mutant of the model legume *Lotus japonicus* affected in the low-affinity nitrate transport system (LATS) and describe the changes produced in the nodulation properties of this mutant in the presence of nitrate. In addition, we also show measurements by qRT-PCR of transcript levels for several nitrate transport, nitrate assimilation and other nitrate-related genes both in the WT and *Ljclo1* mutant plants.

MATERIAL AND METHODS

Plant material, growth conditions and nodulation assays

Seeds of *Lotus japonicus* cv. Gifu (GIFU B-129-S9) were initially obtained from Prof. Jens Stougaard (University of Aarhus, Denmark). Plants were grown in a Sanyo SGR193.S26A growth chamber and at 20/18 °C light/dark temperatures and 70 % humidity under a 16 h/8 h light/dark regime. Light intensity at the plant level was 250 µmol m^{-2} s^{-1}, supplied by white fluorescent Philips Colour 83 lamps combined with tungsten lamps to provide red supplement and dawn-dusk effects. Seeds were first scarified by a gentle treatment with Emery paper and then sterilised in 75 % (v/v) ethanol for 5 min and 0.5 % (w/v) hypochlorite for 20 min, followed by several washes with sterile distilled water for one day. Germination and growth were carried out in pots (five plants per pot). Unless otherwise stated the standard nutrient solution used for daily irrigation of seed trays was "Hornum", as described by Handberg & Stougaard (1992). This medium uses 3 mM KNO_3 and 5 mM NH_4NO_3 as N sources (mixed nutrition, N-sufficient conditions). In some cases, either KNO_3 or NH_4Cl at the concentrations indicated were used as the single nitrogen source. 3 mM KCl was added in ammonium media or -N to compensate for the lack of K.

For nodulation studies *M. loti* strain TONO JA76 (Kawaguchi *et al.* 2002) was grown in YM liquid medium (Vincent, 1970) at 28ºC up to an OD at 600 nm = 1, then collected by centrifugation for 30 min at 2,408 x *g* and resuspended in 0.75% (w/v) NaCl. Once sown in the pots, the plants were inoculated by addition of 2 ml of this bacterial solution. A control with non-inoculated plants was carried out to check for the absence of nodules. For some particular experiments different concentrations of KNO_3 were used.

Selection of *Ljclo1* chlorate resistant mutant from *L. japonicus*

The *Ljclo1* mutant was selected after mutagenic treatment with EMS from the M2 or M3 (100,000 plants) progenies of 6,100 M1 plants (Márquez *et al.* 2005). M2 or M3 plants were grown in greenhouse on Hornum medium (3 mM KNO_3 and 5 mM NH_4NO_3) using vermiculite or terragreen as solid support for 30 days. After plants were irrigated with distilled water for 48 h, they were irrigated with Hornum base medium supplied with 1 or 5 mM $KClO_3$ and 5 mM KNO_3. 250 putative chlorate resistant plants were selected and rescued in Hornum medium. One particular chlorate-resistant plant that showed 100% inheritance of this character on its progeny was named *Ljclo1* and was the subject of the studies shown in this paper.

Uptake measurements using depletion assays

One-month-old plants were transferred from vermiculite to hydroponic cultures containing ¼ Hornum media with some modifications (0.5 mM $CaCl_2$, 0.2 mM $CaSO_4$, 0.2 mM KNO_3, no ammonia) for 5 days. The net NO_3^- uptake was calculated by following depletion from the uptake solution containing 0.5 mM $CaSO_4$ and 0.15 or 2 mM KNO_3, for HATS and LATS measurements respectively. The nitrate concentration of the uptake solution was measured spectrophotometrically as described previously (Pal'ove-Balang & Mistrík 2002). The medium for chlorate uptake determinations contained 0.5 mM $CaSO_4$ and 1 mM $KClO_3$. The depletion of chlorate was measured spectrophotometrically according to method of Doddema *et al.* (1978).

Electrophysiological measurements

One-month-old plants were transferred to hydroponics for 1 day. For nitrate uptake measurements the hydroponic solution contained 0.5 mM $CaSO_4$ and 0.1 mM $Ca(NO_3)_2$. Roots were cut (25 mm long segments) and attached to a Plexiglas holder and mounted in a vertical 5 cm^3 cuvette which was perfused with solution containing 1 mM KCl, 0,25 mM $MgSO_4$, 0.1 mM NaH_2PO_4 and 0.5 mM $CaSO_4$ (pH 5,7). Nitrate was added as 5 mM $Ca(NO_3)_2$ to the same solution. For chlorate uptake measurements the hydroponic solution contained 0.5 mM $CaSO_4$ and 0.2 mM $NaNO_3$. Roots were treated as previously and the cuvette was perfused with solution containing 1 mM KCl, 0.25 mM $MgSO_4$, 0.1 mM NaH_2PO_4, 0.1 mM $CaSO_4$ and 2.5 mM Na_2SO_4. Chlorate was added by replacing the 2.5 mM Na_2SO_4 with 5 mM $NaClO_3$. Measurements of the membrane potential (Em) were carried out at 22°C, using standard microelectrode

techniques which have been described in detail by Pavlovkin *et al.* (1993). Measurements were performed in the outermost layer of the root cortex 2 mm behind the root cap.

Nitrate, chlorophyll and enzyme assays determinations

Colorimetric determinations of nitrate content in root crude extracts were performed as described by Cataldo *et al.* (1975), with slight modifications (Pajuelo *et al.* 2002): 200 □l of 5 % (p/v) salicylic acid dissolved in 96 % (p/v) sulphuric acid was added to aliquots of 50 □l from the root crude extracts and left to react for 20 min. 4.75 ml of 2 M NaOH was added to the reaction mixtures and then the absorbance was read at 405 nm after cooling. A calibration curve of known amounts of nitrate dissolved in the standard extraction buffer was used for analytical determinations. Controls were set up without salicylic acid.

Chlorophyll content was determined according to Arnon (1949). Shoots from 15 days old *L. japonicus* plants were ground in a mortar in presence of liquid nitrogen. Then, 20 ml of 80 % (v/v) acetone were added to 1 g of ground material and shaken vigorously. The homogenates were centrifuged for 10 min at 15,000 x *g* and insoluble material was removed. The absorbance was read at 652 nm in supernatants.

NR enzyme assays were carried out as described previously (Pajuelo et al. 2002).

RNA extraction and qRT-PCR measurements

Leaf or root material was flash frozen in liquid nitrogen, homogenized with a mortar and pestle, and kept at −80°C until use. Three independent biological replicates were used for the transcriptomic and quantitative real-time RT-PCR (qRT-PCR) analyses as well as for metabolite profiling analysis. Total RNA was isolated using the hot borate method (Sánchez *et al.* 2008). The integrity and concentration of the RNA preparations were checked using an Experion bioanalyzer (Bio-Rad, http://www.bio-rad.com) with RNA StdSens chips and a Nano-Drop ND-1000 (Nano-Drop Technologies, http://www.nanodrop.com), respectively.

qRT-PCR analysis was carried out as described elsewhere (Díaz *et al.* 2010; Pérez-Delgado *et al.* 2013). For qRT-PCR analysis, total RNA was treated with the TURBO DNA-free DNAse (Ambion). Reverse transcription was carried

out using SuperScript III reverse transcriptase (Invitrogen), OligodT and RNAsin RNAse inhibitor (Ambion). DNA contamination and RNA integrity were checked by carrying out qRT-PCR reactions with oligonucleotides that amplified an intron in the *L.japonicus* Hypernodulation Aberrant Root (*LjHAR1*) gene and the 3' and 5' extremity of the *L. japonicus* glyceraldehyde-3-phosphate dehydrogenase respectively. qRT-PCR reactions were carried out in 10 µL in a Lightcycler 480 thermal cycler (Roche) using a SensiFAST SYBR No-ROX Kit (Bioline). Expression data were normalized using the geometric mean of four housekeeping genes: *L. japonicus* glycosylphosphatidyl inositol (*LjGPI)*-anchored protein (chr3.CM0047.42), *L. japonicus* protein phosphatase 2A (*LjPP2A;* chr2.CM0310.22), *L. japonicus* ubiquitin carrier protein 10 (*LjUBC10*; chr1.TM0487.4), and *L. japonicus* polyubiquitin 4 (*LjUBQ4*; chr5.CM0956.27), that were selected amongst the most stably expressed genes in plants (Czechowski et al. 2004). A list of all the oligonucleotides used is provided in Supplemental Table S1. Sequence data and nomenclature that is used in this article can be found in the Kazusa 2.5 database (www.kazusa.or.jp/lotus/).

RESULTS

Characterization of growth and chlorophyll content in *Ljclo1* mutants

Fig. 1 shows the typical picture of *Ljclo1* mutant used in the present work. This mutant was rescued from a chlorate resistance screening carried out previously (Márquez et al. 2005). As shown in Fig. 1, mutant plants were able to grow quite satisfactorily for one week under the presence of chlorate concentrations ranging 0.5-10 mM $KClO_3$, while these conditions killed the wild type *L. japonicus* plants.

Growth parameters of WT and *Ljclo1* mutant were determined under the presence of different nitrogen nutrition regimes in the absence of chlorate. The results obtained are summarised in Fig. 2. Roots and shoots from mutant plants showed about 60 % inhibition in terms of fresh weight when the plants were grown in the presence of nitrate as a single nitrogen source, in comparison with the WT.

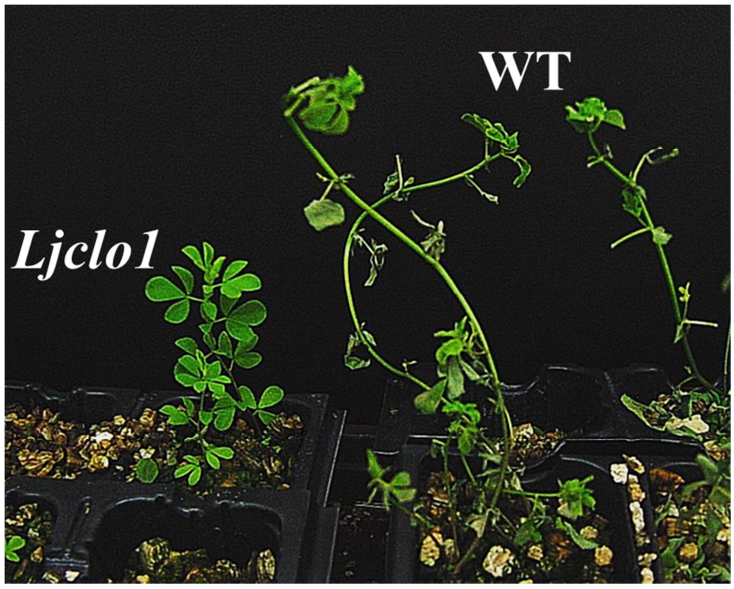

Fig. 1. Phenotype of the *Ljclo1* chlorate resistant mutant analysed in this work, compared to WT plants, after 5 days in the presence of 5 mM $KClO_3$ plus 5 mM KNO_3.

The inhibition of growth in the mutant plants was significantly lower when the plants were grown under mixed ammonium plus nitrate nutrition. In contrast, no significant difference was observed between WT and mutant plants when the plants were grown under ammonium as the single nitrogen source, in spite of the fact that growth in ammonium for the WT was much lower than in the presence of nitrate. All the differences observed in fresh weight of roots (Fig. 2A) and shoots (Fig. 2B) were also confirmed in dry weight determinations (Figs. 2C and 2D, respectively).

Interestingly, some differences were observed with regard to the younger leaves of *Ljclo1* mutant plants which looked pale green when compared to the WT. Fig. 3 shows that the chlorophyll content of the leaves from the shoot tip of the mutant plants was about 50 % lower than for the WT in the same growing conditions in the presence of nitrate. However, no significant difference to the WT was observed with regard to the chlorophyll content of the lower (mature) leaves (Fig. 3).

Considering that the detrimental effects on growth of *Ljclo1* plants were mostly noticeable in the presence of nitrate it was therefore hypothesised that *Ljclo1* mutant could be affected in nitrate uptake or utilisation.

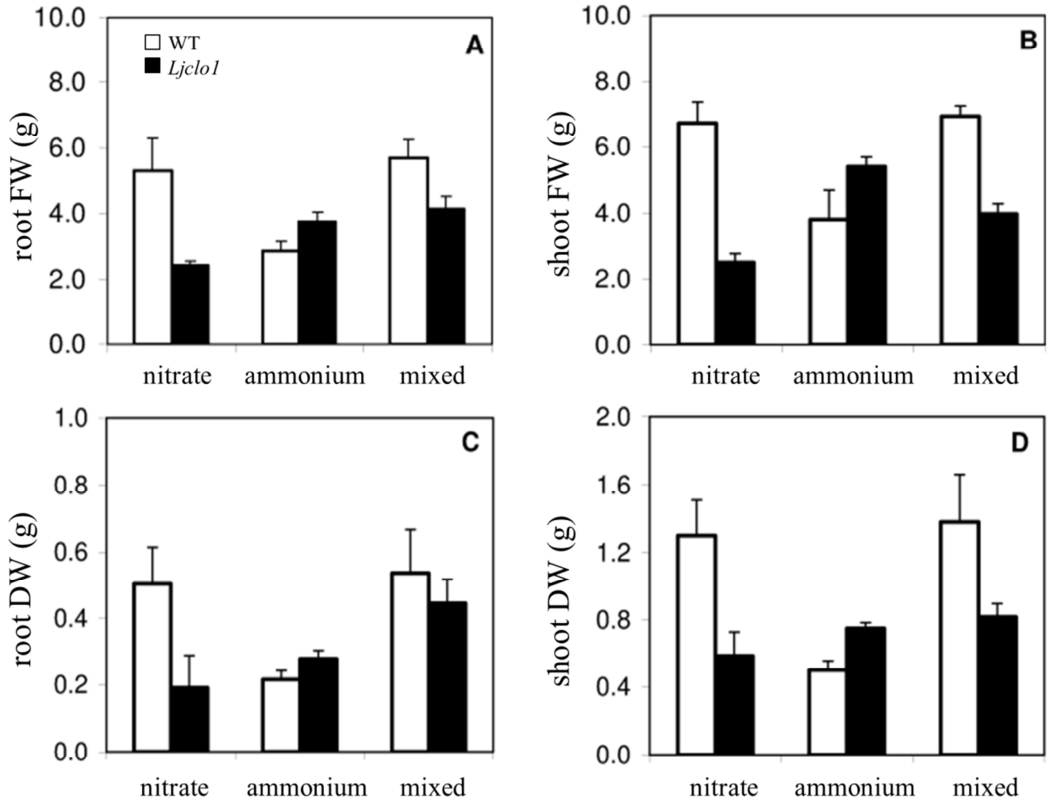

Fig. 2 Comparison of fresh and dry weight of roots (A,C) and shoots (B,D) of 2-month old WT and *Ljclo1* plants grown on different nitrogen sources. Plants were grown on standard Hornum media containing 5 mM NH_4NO_3 and 3 mM KNO_3 (mixed), or 8 mM NH_4Cl (ammonia) or 8 mM KNO_3 (nitrate) as the N source. The results are the means of 6 different plants (± SE) for FW and 3 different plants for DW.

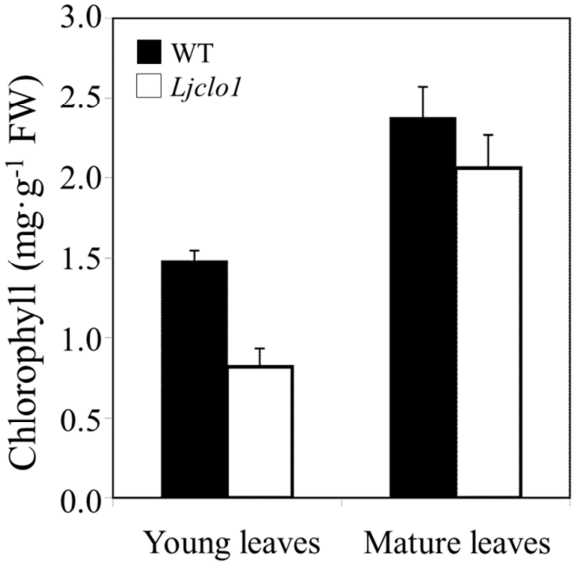

Fig. 3. Difference in chlorophyll content in young or mature leaves from *Ljclo1* mutant plants, compared to the WT.

Nitrate reductase and nitrate / chlorate uptake measurements

According to present knowledge, chlorate resistance in higher plants can be caused mainly by two different mechanisms: by reduced reduction of chlorate to chlorite due to a mutation on NR or related genes, or a reduced increase in tissue chlorate content caused by a mutation of a nitrate transporter gene with some affinity for chlorate (Navarro et al. 2005 and references therein). In the case of *Ljclo1* mutant plants it was determined that total NR activity and nitrate content of the plants were not significantly different to the WT (results not shown). Therefore, the uptake of nitrate was analysed at 0.15 and 2 mM external nitrate concentrations in order to characterise either the high- or low-affinity nitrate uptake systems. At low nitrate concentration (0.15 mM) it was not possible to observe any significant difference in nitrate uptake rate between the WT and *Ljclo1* mutant (Fig. 4A). However, nitrate uptake was strongly reduced in *Ljclo1* plants in high (2 mM) external nitrate concentration (Fig. 4B). In addition, it was also determined that the uptake of chlorate observed in *Ljclo1* plants in the presence of 1 mM chlorate was less than half of that obtained for the WT (Fig. 4C). Therefore it could be concluded that the *Ljclo1* mutant may be deficient in a low-affinity nitrate transport system which can be also used for chlorate uptake, thus explaining the chlorate resistance phenotype.

A series of electrophysiological measurements were also carried out in order to confirm the nitrate and chlorate uptake defects of *Ljclo1* mutant plants (Fig. 5). A rapid electrical response of *Lotus* cortical root cells to nitrate and chlorate was observed. Nitrate, applied as 5 mM $Ca(NO_3)_2$, caused a membrane depolarisation of ΔE_M = 51 mV ± 0.48 (SE) in WT and 28 mV ± 1.03 in *Ljclo1* (Fig. 5, top) (experiment was repeated four times). The same kind of experiment was also performed by replacing the nitrate by 5 mM $NaClO_3$, which caused a membrane depolarisation by ΔE_M = 20 mV ± 1.9 (SE) in WT and 9 mV ± 1.04 in *Ljclo1*. (Fig. 5,bottom). In conclusion, the electrophysiological results confirmed that the uptake rate of nitrate (and chlorate) was reduced in *Ljclo1* mutants.

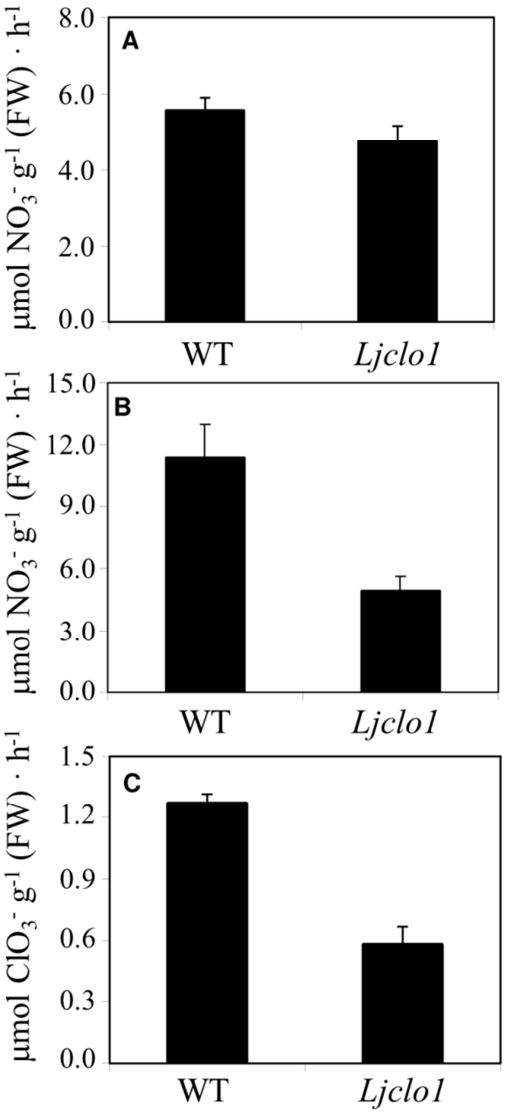

Fig. 4. Nitrate (A,B) and chlorate (C) uptake by WT and *Ljclo1* mutant plants. Measurements of nitrate and chlorate uptake were done by nitrate or chlorate depletion from the medium as described in Materials and Methods. Nitrate depletion was determined in solutions containing either 0.15 mM (A) or 2 mM (B) external nitrate concentrations for measurements of HATS (0.15 mM) or LATS (2 mM) respectively. Chlorate uptake was determined in media containing 1 mM $KClO_3$. The results are the means (± SE) of three experiments (each containing two replicates).

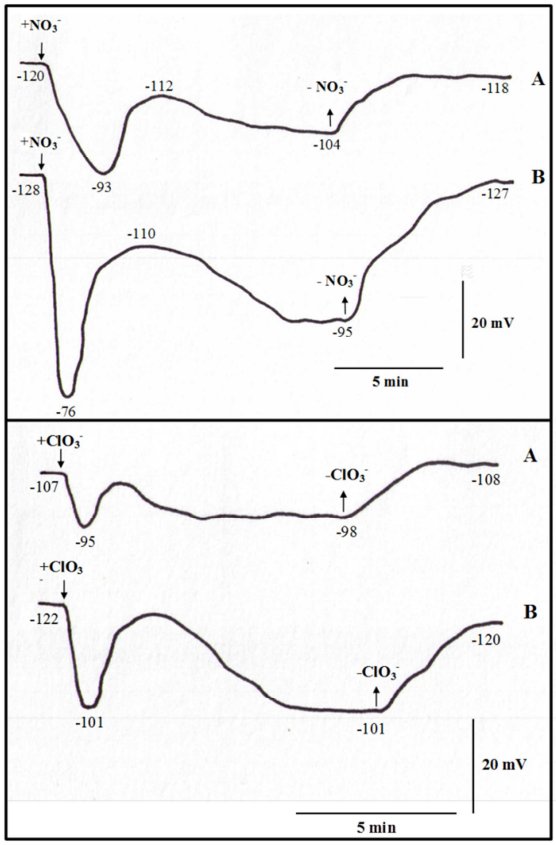

Fig. 5. Electrophysiological measurements of nitrate and chlorate uptake**.** Plasma membrane potential measurements of outer cortex cell, 2 mm from the tip of *Lotus* roots. Effect of the 5 mM nitrate (top panel) or 5 mM chlorate (bottom panel) addition/withdrawal on the electrophysiological signals obtained for *Ljclo1* mutant (trace A) compared to the WT (trace B). Each experiment was repeated 4 times, and one representative result is shown. Recorded values are in mV. Other details of these experiments are given in Materials and Methods.

All these results suggest that the mutation produced in *Ljclo1* may be related to some of the LATS nitrate transporters, or to any of the regulatory mechanisms that may be related to nitrate/chlorate uptake.

The inheritance of all the different phenotypes observed for *Ljclo1* plants was examined throughout the progeny of a back-cross of *Ljclo1* (♀) x WT (♂). A 3:1 segregation ratio for the inheritance of a single Mendelian recessive trait was obtained in all cases, including nitrate uptake, electrophysiological and plant growth measurements. This is shown in supplemental Fig. S1 where a clear co-segregation of the different mutant characters can be observed among the progeny.

Nodulation studies

Table 1 shows the results of different nodulation studies carried out in WT and *Ljclo1* mutant plants, both in the presence or absence of nitrate and at two different times after inoculation with *Mesorhizobium loti*. Total number and fresh weight of nodules, as well as the specific ratios of nodule fresh weight per nodule number and nodule fresh weight per root fresh weight were determined in nodulation studies carried out in the absence of any added nitrogen source , or in the presence of 0.5 mM, 1 mM or 2 mM nitrate. It was necessary to allow for any possible reduced growth of *Ljclo1* plants that could be taking place in the presence of nitrate, as described above. Therefore, nodulation parameters were determined at 40 dpi and 60 dpi for the WT, and 50 and 70 dpi for the *Ljclo1* mutant, respectively, in order to compare WT and mutant plants with a similar size and developmental stage. The ratios of fresh weight of nodules to fresh weight of roots was considerably lower both in the WT and *Ljclo1* mutant plants at all nitrate concentrations tested when compared to nodulation tests carried out in the absence of nitrate. This decrease in the ratios of nodules was higher at higher nitrate concentrations in both genotypes. The comparison of the ratios of fresh weight of nodules to fresh weight of roots among WT and *Ljclo1* plants showed no significant differences at either 40-50 or 60-70 dpi when nodulation took place either in the absence of any added exogenous nitrogen or in the presence of 0.5 mM NO_3^-. In contrast, several differences in the specific ratios of fresh weight of nodules per fresh weight of roots were observed among WT and *Ljclo1* plants at higher tested NO_3^- concentrations (1 and 2 mM). The results obtained in mutant plants indicated that reduced nodulation was produced in the presence of 1-2 mM nitrate in the *Ljclo1* mutants compared to the wild type. In fact, no visible nodules were observed at 50 dpi in the mutant plants in the presence of either 1 mM or 2 mM nitrate, while some nodulation was still detectable in the WT (Table 1). Furthermore, at 60-70 dpi, approximately 70% reduction in the ratio between fresh weight of nodules and fresh weight of roots was observed in the *Ljclo1* mutant at 1 mM NO_3^-, compared to nodulation in the absence of exogenous nitrate, while this inhibition was only about 30% in the WT (Table 1, 60-70 dpi). In addition, it was also observed that the low number of nodules still present at 2 mM NO_3^- for the mutant plants at 70 dpi was almost the half than those of the WT at 60 dpi.

Measurements of transcript levels in WT and *Ljclo1* mutants

A wide range of nitrate-related gene transcripts were analysed by qRT-PCR in both WT and *Ljclo1* mutant plants. A total of 35 genes were examined

which comprised genes encoding for nitrogen assimilatory enzymes (NR, NiR, cytosolic and plastidic GS, Fd- and NADH-GOGATs), as well as the members of NPF and NRT2 nitrate transporters families recently described by Criscuolo *et al.* (2012), together with other nitrate transporters or regulatory genes found in the Kazusa database that have been shown to be nitrate responsive in *Lotus* or other plant species. The basal levels of expression for the different genes analysed was determined in leaves and roots of both the wild type and *Ljclo1* mutants under standard N-sufficient conditions, and the results obtained are shown in Table 2.

			nod.number/pl.	FW nod.(mg)/pl.	FW nod.(mg)/ nod.number	FW nod./FW root
40/50 dpi	- NO_3^-	WT	15.30 ± 1.55*	11.50 ± 1.44	0.762 ± 0.070*	0.435 ± 0.043
		Ljclo1	23.83 ± 2.41	11.43 ± 1.33	0.500 ± 0.075	0.440 ± 0.053
	0.5mM NO_3^-	WT	18.75 ± 4.43	6.75 ± 2.06	0.350 ± 0.017*	0.127 ± 0.031
		Ljclo1	20.44 ± 3.54	15.50 ± 3.50	0.710 ± 0.058	0.188 ± 0.023
	1 mM NO_3^-	WT	22.33 ± 3.61	9.00 ± 1.83	0.390 ± 0.043	0.076 ± 0.016
		Ljclo1	-	-	-	-
	2 mM NO_3^-	WT	-	-	-	-
		Ljclo1	-	-	-	-
60/70 dpi	- NO_3^-	WT	57.67 ± 6.20	99.56 ± 14.21	1.698 ± 0.133	0.589 ± 0.036
		Ljclo1	47.60 ± 6.49	64.80 ± 15.71	1.303 ± 0.197	0.655 ± 0.026
	0.5mM NO_3^-	WT	70.10 ± 8.87	117.40 ± 24.33	1.510 ± 0.196	0.506 ± 0.026
		Ljclo1	64.50 ± 8.00	97.25 ± 20.65	1.407 ± 0.166	0.482 ± 0.019
	1 mM NO_3^-	WT	81.89 ± 5.12*	151.00 ± 19.19*	1.830 ± 0.175*	0.430 ± 0.018*
		Ljclo1	34.67 ± 3.87	20.84 ± 4.81	0.569 ± 0.087	0.171 ± 0.023
	2 mM NO_3^-	WT	38.50 ± 8.45*	20.75 ± 6.84*	0.490 ± 0.075	0.080 ± 0.017*
		Ljclo1	15.00 ± 2.45	5.80 ± 1.23	0.377 ± 0.200	0.035 ± 0.007

Table 1. Nodulation parameters of *Lotus japonicus* WT and *Ljclo1* mutant plants in the absence or presence of different concentrations of nitrate. The table shows the nodulation parameters obtained from plants of WT and *Ljclo1* at two different times after inoculation with *M. loti* (40 dpi and 60 dpi for the WT and 50 and 70 dpi for *Ljclo1* mutant respectively) to allow for any possible lower growth of *Ljclo1* plants in the presence of nitrate). Different columns show the total nodule number per plant, total fresh weight of nodules per plant, ratio between the fresh weight of nodules to the nodule number and ratio of the fresh weight of nodules to the fresh weight of roots. Values are the means for three determinations for each of three biological replicates ± standard error. * indicates significant difference between WT and *Ljclo1* mutant in each condition as determined by Student test ($P<0.05$). - indicates that no significant nodulation was detected.

In general very important changes were not observed in the levels of expression of those genes examined in *Ljclo1* plants compared to the WT. Several of the genes showed very high levels of expression in both genotypes in either leaves and in roots, for example, genes for NR (*LjNIA),* NiR (*LjNII*), cytosolic GS1.2 (*LjGLN1.2),* plastid GS *(LjGLN2*) and Fd-GOGAT *(LjGLU1).* Among nitrate transporters, one of the members of the *NPF* family (*chr2.CM0826.350,* a close homologue to *MtNPF6.8*) showed its highest level of

expression in leaves, while another one (*chr4.CM247.130*, a close homologue to *AtNPF7.3*) was also relatively abundant both in leaves and in roots. With regard to the *NRT2* family, *chr3.CM0649.30* (also named *LjNRT2.2*) was the most highly expressed in roots.

The main relevant differences observed in the expression of the genes analysed in WT and *Ljclo1* mutants are shown in Table 2. Several genes were about 2-fold more highly expressed in leaves of *Ljclo1* than in the WT leaves, such as those for plastid GS (*LjGLN2*), Fd-GOGAT (*LjGLU1*) and NiR (*LjNII)*. In contrast, other genes showed more than 2-fold reduction in expression in *Ljclo1* compared to the WT, either in leaves (such as *LjGLN1.2* and *LjGLN1.3*, both encoding for cytosolic GS, and *chr4.CM0161.180* a transporter closely homologous to *AtNRT2.5*) or in roots (such as genes for transporters *chr2.CM0826.350*, *LjB20H09.30* and *chr1.CM0001.20*).

Type of gene (gene name or family)	Kazusa 2.5 code (closest homologue)	Level of expression (relative units)					
		WT		*Ljclo1* mutant			
		Leaves	Roots	Leaves	%	Roots	%
A (*LjNIA)*	*LjT01E03.110.r2.m*	4.8433 ± 0.3269	4.1762 ± 0.3990	4.5433 ± 1.0871	93.8	3.5727 ± 0.8438	85.5
A (*LjNII)*	*chr4.CM0227.280.r2.d*	1.2099 ± 0.2276	2.5651 ± 0.1675	2.9407 ± 0.4650	**243.0**	2.2718 ± 0.2602	88.6
A (*LjGLN1.1)*	*chr2.CM0312.1480.r2.m*	0.1860 ± 0.0667	0.8656 ± 0.1054	0.2110 ± 0.0152	113.5	0.8686 ± 0.0712	100.4
A (*LjGLN1.2)*	*chr6.CM0014.300.r2.m*	3.5210 ± 1.3755	5.9084 ± 1.0915	1.3166 ± 0.2783	**37.4**	6.4865 ± 0.4366	109.8
A (*LjGLN1.3)*	*LjSGA_030247.1*	0.0506 ± 0.0165	0.0010 ± 0.0002	0.0243 ± 0.0012	**48.0**	0.0010 ± 0.0005	93.8
A (*LjGLN1.4)*	*LjSGA_058827.1*	0.0003 ± 0.0001	0.0006 ± 0.0001	0.0004 ±0.0001	125.2	0.0009 ± 0.0004	139.5
A (*LjGLN1.5)*	*LjSGA_019428.1*	0.0034 ± 0.0024	n.d.	0.0009 ± 0.0008	25.9	n.d.	n.d.
A (*LjGLN2)*	*chr6.CM0139.890.r2.m*	6.6710 ± 1.2614	1.3161 ± 0.0257	16.1139 ± 0.9837	**241.6**	1.4585 ± 0.1112	110.8
A (*LjGLU1)*	*chr1.CM0009.170.r2.d*	2.6475 ± 0.2080	0.9873 ± 0.1013	5.1697 ± 0.0709	**195.3**	0.8750 ± 0.0845	88.6
A (*LjGLT1)*	*LjSGA_035611.1*	0.0070 ± 0.0017	0.0175 ± 0.0068	0.0057 ± 0.0009	82.2	0.0173 ± 0.0023	99.3
A (*LjGLT2)*	*LjSGA_037992.1*	0.1333 ± 0.0219	0.4032 ± 0.0905	0.1253 ± 0.0213	94.0	0.3730 ± 0.0456	92.5
T (*NPF*)	*chr2.CM0021.2180 (AtNPF6.3)*	0.1035 ± 0.0108	0.8377 ± 0.1950	0.0908 ± 0.0149	87.7	0.5588 ± 0.0631	66.7
T (*NPF*)	*chr2.CM0021.2200 (At NPF6.3)*	0.0612 ± 0.0227	0.0002 ± 0.0002	0.0851 ± 0.0145	138.9	0.0004 ± 0.0001	156.2
T (*NPF*)	*chr2.CM0021.3040 (At NPF6.3)*	0.1450 ± 0.0215	0.0039 ± 0.0005	0.1978 ±0.0014	136.6	0.0033 ± 0.0010	83.8
T (*NPF*)	*chr2.CM0608.1210*	0.3784 ± 0.0244	0.4697 ± 0.0227	0.3078 ± 0.0316	81.3	0.3593 ± 0.0264	76.5
T (*NPF*)	*chr2.CM0608.1290*	0.0762 ± 0.0158	0.0084 ± 0.0011	0.0633 ± 0.0063	83.0	0.0060 ± 0.0002	72.1

T (*NPF*)	*chr2.CM0826.350 (MtNPF6.8)*	6.0743 ± 0.3659	0.0515 ± 0.0217	7.8989 ± 0.7567	130.0	0.0165 ± 0.0032	**32.1**
T (*NPF*)	*chr2.CM0826.370*	0.0273 ± 0.0024	0.0147 ± 0.0058	0.0221 ± 0.0012	80.9	0.0124 ± 0.0008	84.5
T (*NPF*)	*chr4.CM0170.40*	0.1802 ± 0.0407	0.1715 ± 0.0061	0.1552 ± 0.0190	86.1	0.1442 ± 0.0130	84.1
T (*NPF*)	*chr4.CM0247.130 (AtNPF7.3)*	1.2997 ± 0.1096	1.1559 ± 0.4087	1.1363 ± 0.1174	87.4	0.8082 ± 0.0287	69.9
T (*NPF*)	*chr4.LjB20H09.30 (AtNPF6.3)*	0.0022 ± 0.0003	0.0023 ± 0.0009	0.0021 ± 0.0006	99.1	0.0011 ± 0.0001	**49.1**
T (*NPF*)	*chr6.CM0118.580*	0.0025 ± 0.0002	0.0015 ± 0.0004	0.0032 ± 0.0018	131.3	0.0014 ± 0.0002	95.7
T (*LjNPF1.7)*	*LjSGA_046844.2*	1.6276 ± 0.3997	0.6440 ± 0.0385	1.6270 ± 0.2917	100.0	0.3518 ± 0.0271	54.6
T (*NRT2*)	*chr1.CM0001.20 (AtNRT2.7)*	0.5153 ± 0.2244	0.0039 ± 0.0008	0.4512 ± 0.0511	87.6	0.0013 ± 0.0003	**33.9**
T (*LjNRT2.1*)	*chr3.CM0649.40*	0.0006 ± 0.0007	0.6645 ± 0.2386	0.0008 ± 0.0004	132.6	0.7314 ± 0.1442	110.1
T (*LjNRT2.2*)	*chr3.CM0649.30*	0.0014 ± 0.0008	9.5941 ± 2.3133	0.0014 ± 0.0012	100.7	6.9253 ± 1.7121	72.2
T (*NRT2*)	*chr4.CM0161.180 (AtNRT2.5)*	0.2712 ± 0.1152	0.0664 ± 0.0242	0.1114 ± 0.0342	**41.1**	0.0840 ± 0.0097	126.5
T (*LjCLCa)*	*chr1.CM0064.1040.r2.m (At5g40890)*	2.2148 ± 0.2224	1.0378 ± 0.3649	2.4472 ± 0.1554	110.5	0.7878 ± 0.1348	75.9
T (*LjN70)*	*LjSGA_018310.1*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
R (*LjLBD38)*	*chr4.CM0128.420.r2.m (At3g49940)*	0.3897 ± 0.0946	0.4208 ± 0.0608	0.8228 ± 0.1502	**211.1**	0.5812 ± 0.0472	138.1
R (*LjCIPK8)*	*LjSGA_048938.1 (At4g24400)*	0.0447 ± 0.0108	0.0412 ± 0.0057	0.0573 ± 0.0035	128.3	0.0352 ± 0.0036	85.5
R (*LjCIPK23)*	*chr6.CM0037.1410.r2.m (At1g30270)*	0.1254 ± 0.0056	0.5649 ± 0.0406	0.0908 ± 0.0100	72.5	0.3455 ± 0.0170	61.2
R (*LjANR1)*	*chr6.CM0055.400.r2.m (At2g14210)*	0.0010 ± 0.0002	0.0035 ± 0.0015	0.0009 ± 0.0003	93.0	0.0066 ± 0.0016	187.3
R (*LjCLE-RS2)*	*chr3.CM2103.25.r2.a*	n.d.	0.0067 ± 0.0025	n.d.	n.d.	0.0087 ± 0.0033	130.2
R (*LjNLP7/NIN)*	*chr5.CM0148.170.r2.a (At4g4020)*	0.3668 ± 0.0251	0.3526 ± 0.1117	0.3097 ± 0.0239	84.4	0.3743 ± 0.0233	106.1

Table 2. Levels of nitrate-related gene transcripts under N-sufficient conditions in WT and *Ljclo1* mutant plant. The first column on the left shows a classification of the genes analysed according to the following: A, genes involved in nitrogen assimilation; T, some of the transporters or ion channels found in *Lotus* or other plant species which may be related to nitrate transport; R, selected regulatory genes shown to be nitrate responsive in *Lotus* or other plant species. The name of genes for *L. japonicus* (*Lj*) analyzed in the table is also indicated in this column in brackets. In the case of transporters characterized by Criscuolo *et al.* (2012) it is indicated in brackets if they were assigned to *NPF* or *NRT2* families. The names of the closest homologous genes from *Arabidopsis thaliana* (At) or *Medicago truncatula* (Mt) are shown in brackets in the second column of the table. Absolute transcript levels are indicated for the WT and *Ljclo1* as determined according to Material and Methods, and, in the case of *Ljclo1*, data are also expressed as % of the values found for each gene on the same tissue in WT plants. Values in bold correspond to the expression levels significantly different in WT and *Ljclo1* (according to Student's t test, $p < 0.05$) that were also in the range of 2-fold or a higher change in *Ljclo1* plants compared to the WT. Non-detectable transcripts are indicated as n.d. Significance of gene names is as described elsewhere (Márquez *et al.* 2005, Masclaux-Daubresse *et al.* 2010, Pérez-Delgado *et al.* 2013) or in references provided: *NIA*, gene encoding for NR*; NII*, gene encoding for NiR; *GLN1*, gene encoding for cytosolic GS (GS1); *GLN2*, gene encoding for plastidic GS (GS2); *GLU1*, gene encoding for Fd-GOGAT; *GLT*, genes encoding for NADH-GOGAT; *NRT*,

nitrate transporter genes (Criscuolo *et al.* 2012); *NPF1.7*, numerous infections and polyphenolics/lateral root organ defective (Bagchi *et al.* 2012); *CLCa*, Chloride channel a (Wang *et al.* 2012); *LjN70*, symbiosome transporter (Vincill *et al.* 2005); *LBD*, Lateral organ boundaries domain (Rubin *et al.* 2009); *CIPK*, Calcineurin B-like-interacting protein kinase (Wang *et al.* 2012); *ANR*, *Arabidopis* nitrate regulated (Forde, 2000); *CLE-RS2*, Clavata3/Endosperm surrounding region root signal 2 (Okamoto *et al.* 2009); *NLP*, nodule inception (NIN)-like protein (Castaings *et al.* 2009). Kazusa 2.5 code corresponds to the code given for each gene in the Kazusa 2.5 database (http://www.kazusa.or.jp/lotus).

No big differences were detected between *Ljclo1* and WT plants with regard to the levels of transcripts found for other nitrate-responsive proteins described in literature including ion channels such as CLCa, kinases such as CIPK8 or CIPK23, regulatory peptides such as LjCLE-RS2 or members of different families of transcription factors (LBD, ANR1, NLP7/NIN), except for a 2-fold increase in *LjLBD38* expression in leaves (Table 2).

The regulatory behaviour of all the nitrate-related genes analysed was also examined in *Ljclo1* mutant plants in comparison with the WT. For this purpose, plants were transferred from N-sufficient to N-starvation conditions for 3 and 10 days, and transcript levels were determined in leaves and roots of these plants. Results from all these measurements are shown in supplemental Fig. S2. From all the genes analysed, different groups of genes could be identified according to their regulatory behaviour in the WT: a) Genes that were down-regulated both in leaves and in roots under N-starvation conditions, which included genes for NR (*LjNIA*), NiR (*LjNII*), plastidic GS (*LjGLN2*), Fd-GOGAT (*LjGLU1*), *chr2.CM0021.2180*, *chr2.CM0021.3040*, *chr1.CM001.20*, *LjNPF1.7*, *LjCLCa* and *LjNLP7/NIN*; b) Genes that were down-regulated under N-starvation in leaves but not in roots: these included *chr2.CM0826.350*, *chr2.CM0021.2200* and *chr3.CM0649.40* (*LjNRT2.1*); c) Genes that were down-regulated under N-starvation in roots but not in leaves: *chr4.LjB20H09.30*, *chr3.CM0649.30* (*LjNRT2.2)*, and *LjCIPK23* (together with *LjCLE-RS2* whose expression was down-regulated in roots but no significant levels were detected in leaves); d) Genes that were up-regulated under N-starvation: this was clearly observed for *chr2.CM0608.1290* in roots. Other genes not mentioned above did not show any clear regulatory trend. It is important to state that most of these patterns of regulation observed in WT plants on transfer to N-starvation conditions were not substantially altered in *Ljclo1* mutant plants for most of the genes analysed, except for some minor differences such as a trend towards a more constitutive expression of *chr3.0649.40* (*LjNRT2.1* transporter) in leaves, and *chr1.CM0001.20* in roots.

DISCUSSION

The isolation of chlorate resistant mutants has been a useful strategy for identifying genes in the nitrate assimilatory pathway (Navarro et al. 2005, and references therein). In this system, the NR enzyme is considered as a key step that mediates the production of the toxic compound chlorite from chlorate. Nitrate transporters can also transport chlorate (Deane-Drummond 1984) and contribute to its toxicity (Prieto & Fernández 1993). Thus, a lower uptake of chlorate may be expected in mutations that affect nitrate uptake (Oostinder-Braaksma & Feenstra 1973, Wilkinson & Crawford 1993, Hoff *et al.* 1994, Huang *et al.* 1996). This was the case for the *Ljclo1* chlorate-resistant mutant described in the present paper. We have shown that this particular mutant has normal levels of NR, but it is affected in LATS for nitrate and chlorate, as could be determined both from nitrate/chlorate uptake analyses and electrophysiological measurements. The large membrane depolarisation occurring during anion transport in *Lotus* roots can be explained by $2H^+/A^-$ cotransport mechanism previously suggested for anion uptake in other plant systems (Miller et al. 2007). However, this nitrate uptake defect observed in *Ljclo1* does not seem to affect the total level of nitrate that can be accumulated in either leaves or roots from this mutant, suggesting that sufficient activity of most of the high- and low-affinity nitrate transport systems must be present . Molecular characterization of the *L. japonicus NPF* and *NRT2* families was recently initiated by Criscuolo *et al.* (2012). Retrieval of *Lotus* sequences from the Kazusa genome sequence resource (http:www.kazusa.or.jp/lotus) led to the identification of 37 putative *Lotus NPF* sequences and 4 putative members of the *NRT2* family, but this does not exclude that other members are present and can be identified at a later stage. A high number of nitrate transporters have also been identified in other plant species. For example, *A. thaliana* and rice have 53 and 80 NPF members respectively, together with a small family of NRT2 proteins comprising 7 and 4 members in *Arabidopsis* and rice, respectively (Tsay *et al.* 2007).

An important restriction in plant growth was also observed in *Ljclo1* plants, which affected both shoot and root organs. It is quite likely that this growth defect may be related to the nitrate uptake deficiency previously commented on. It is well known that besides its role as a nutrient, NO_3^- also acts as a signal molecule that controls many aspects of plant metabolism and development, including gene transcription, protein accumulation, phosphorylation events, germination, stomatal movements, and shoot and root development (Gojon *et al.* 2011, and references therein). However, the signalling

pathways activated by NO_3^- sensing and triggering of various responses to nitrate are very complex and far from being completely characterized, in spite of this being a very active area of research (Gojon et al. 2011). Despite progress in understanding the nature of local nutrient signaling, the signaling mechanisms and the long-distance (systemic) signals through which a plant regulates root activity according to its nutrient status remain largely unknown (Ruffel *et al.* 2011). Therefore, it is not possible at this stage to define more precisely the specific molecular lesions that may be affecting *Ljclo1* mutant plants, and how these may resultin the defects in plant growth observed.

Nevertheless, the availability of a *L. japonicus* mutant deficient in nitrate uptake, made it possible for us to analyse the possible interconnection between this defect and the nitrate response to nodulation in this legume plant. It was found that the total number and fresh weight of nodules per plant, as well as the specific ratio of fresh weight of nodules to fresh weight of roots, were all considerably reduced in *Ljclo1* plants compared to the WT in the presence of 1-2 mM nitrate, but not at lower concentrations of nitrate (0.5 mM) or in the absence of external nitrogen. A possible interconnection between *Ljclo1* LATS nitrate uptake deficiency and the observed defect in the nitrate response to nodulation was further supported by the fact that this effect was nitrate concentration dependent and could be mainly observed at the highest range of nitrate concentrations tested (1-2 mM). The differential results obtained on nodulation of *Ljclo1* plants in the presence of nitrate could be indicative that there is high level of internal N satisfaction of *Ljclo1* plants, and this could account for the lower demand for nodules of this plant at 1-2 mM nitrate when compared to the WT plants. The lower growth rate observed for *Ljclo1* plants could be compensating the nitrate uptake deficiency of the plants to result in a high level of N satisfaction of the mutant plants.

The first observations on the inhibitory effect of different nitrogen sources on nodulation were made a long time ago. A model based on a satiety signal that would be translocated from the shoots to the roots and leading to the down-regulation of NO_3^- transport systems has been proposed (Imsade & Touraine 1994). In particular, the effect of nitrate has been extensively studied and the inhibitory action on nodule initiation seems to act locally, whereas both a systemic and local nitrate action was reported for nodules growth and N fixation activity (Omrane & Chiurazzi 2009, and references therein). The existence of a negative feedback that is controlled by the general nutritional status of the plant has been postulated (Omrane & Chiurazzi 2009). However, little is known yet about the genes involved in the long-distance control of root NO_3^- acquisition by

the N status of the plant (Ruffel *et al.* 2008). Recent reports have established that the adaptation of *M. truncatula* to nitrogen limitation is modulated via local and systemic nodule developmental responses (Jeudy *et al.* 2010). On the other hand, it was also shown that symbiotic competence in *L. japonicus* is affected by plant nitrogen status (Omrane *et al.* 2009). Although a variety of regulatory mechanisms are known to be involved in the nitrogen-dependent modulation of the nodule organogenesis program in legume roots, the mechanisms underlying this control are not elucidated yet. In addition, the mechanisms and factors in both local and systemic N supply inhibition as well as the potential targets of their action on the pathways leading to nodule initiation are almost completely unknown (Omrane & Chiurazzi 2009).

An experiment was performed to test whether the transcript levels found in *Ljclo1* mutants under N-sufficient conditions were really reflecting some level of apparent N-satisfaction in these mutant plants. For this purpose measurements of transcript levels were carried out for 35 different genes involved in nitrate transport, assimilation or regulation, both in WT and *Ljclo1*, under N-sufficient (Table 2) and N-starvation conditions (supplemental Fig. S2). Most genes that were down-regulated by N-starvation in WT plants did not show lower levels of transcripts in *Ljclo1* mutants compared to the WT under N-sufficient conditions. On the contrary, several of the genes that were down-regulated under N-starvation in the WT were expressed at slightly higher levels in *Ljclo1* plants (such as those for NiR, plastidic GS and Fd-GOGAT). These results would confirm the apparent N-satisfaction of the mutant plants under N-sufficient conditions and their nitrate response to nodulation.

The fact that no major alterations in transcript levels were found in *Ljclo1* mutants compared to the WT, either under N-sufficient or N-starvation conditions, suggests that most aspects of nitrogen metabolism are not seriously affected in this mutant plant, in spite of the LATS nitrate uptake deficiency. However, we consider that some of the differences observed in transcript levels among *Ljclo1* mutant and WT plants (in general no more than 2-fold higher o lower) may reflect some kind of metabolic compensation operating in *Ljclo1* plants to adapt to the lower nitrate uptake capability of the mutants with a reduced growth rate in mutant plants. In fact, it was of interest to notice that under N-sufficient conditions, the transcript levels for some particular isoforms of cytosolic GS (*GLN1.2 and GLN1.3*) were reduced while those of the plastidic isoform of GS (*GLN2*) and Fd-GOGAT *(GLU1*) were increased and clearly nitrogen responsive. The central role of GS in many aspects of plant growth and productivity of the plants is well known, and our own recent work has proved the

particular significance of plastid GS in the C/N balance of *L. japonicus* plants (Betti *et al.* 2012; Pérez-Delgado *et al.* 2013).

The results shown in the present paper provide valuable quantitative data concerning the absolute levels of transcripts of different NPF and NRT2 transporters, as well as other nitrate-responsive genes under N-sufficient and N-starvation conditions in *L. japonicus* plants. These measurements complement other information available on nitrate transporters in *L. japonicus* (Criscuolo *et al.* 2012). To our knowledge, *Ljclo1* mutants are the first nitrate uptake mutants available in *L. japonicus.* We hope the present results and the availability of *Ljclo1* mutants will encourage more work to increase knowledge about nitrate transporters and nitrate response to nodulation in this particular model legume.

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SUPPORTING INFORMATION

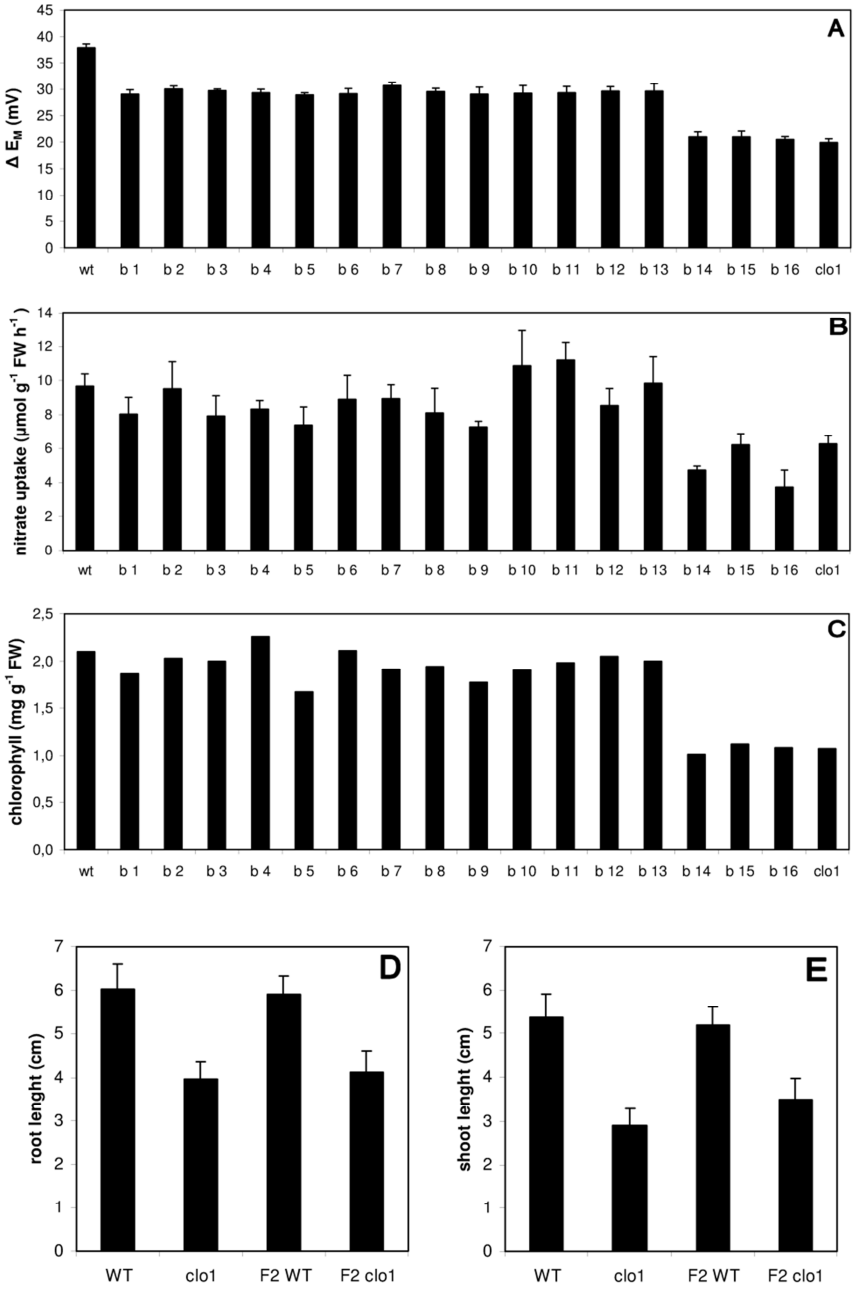
Supplemental Table. S1:

Gene name or family	Kazusa 2.5 code	Primers	
		Forward	Reverse
LjNIA	*LjT01E03.110.r2.m*	GCCAATGCCAATGCATCATCCC	TCGTCACGGAGGGTGTTATGAG
LjNII	*chr4.CM0227.280.r2.d*	AGCAGATGAGATGGACGAGCTG	ACAGTGAGCCTCAGTTCACCAG
LjGLN1.1	*chr2.CM0312.1480.r2.m*	TGGACCACAGGGCCCATAC	AATGTCACGCCCATAGGCTTT
LjGLN1.2	*chr6.CM0014.300.r2.m*	TGAGGTGTGGGTTGCTCGTT	AAGGACCACCCCAGCAATCT
LjGLN1.3	*LjSGA_030247.1*	TAACCTCTCCGAGACCACCG	CCAATCCATATGTATTCGGCG
LjGLN1.4	*LjSGA_058827.1*	AGAGAGACTGAGAAAGATGGAA AATGT	CAGGCCTCCTCTGTCCTCAA
LjGLN1.5	*LjSGA_019428.1*	GGGTAGGCAGGGAGACTGAAA	GAAGCTGGCCTCCTGTCCTC
LjGLN2	*chr6.CM0139.890.r2.m*	GGAAGAGGGAGGCTTTGAGGT	CTGGTGGCGAAGGGATAGATT
LjGLU1	*chr1.CM0009.170.r2.d*	ATTCAACGAGTAACAGCGCCA	CATGGGCTTCAATAAGGCTTCTC
LjGLT1	*LjSGA_035611.1*	TGGTGCTGATGCTATATGCCC	GTCAACCTGCAGTCGCCAA
LjGLT2	*LjSGA_037992.1*	TTGGCAATGTGGCACTGTATG	GCTGCCATCCCGTTGAAAT
NPF	*chr2.CM0021.2180*	CCCTGACCCAACAAAGAAGAAC TG	CAAAGGCGAAGATGTCACTGAGC
NPF	*chr2.CM0021.2200*	AATATCGTCGTCTTTGCCGC	CGTACCTTCCATGCATTTTGG
NPF	*chr2.CM0021.3040*	GCGGTTTTGAGTGGTTTGGA	GGAACGGAGGGAGTAATAAAGAA
NPF	*chr2.CM0608.1210*	GTGTTTGCTGAGTGGGTTGA	CCTTTTGAAGAATGGCTTGG
NPF	*chr2.CM0608.1290*	TGAGGGAGCAAAATTGTGTG	AAATAAATTCGTGCAACACATGA
NPF	*chr2.CM0826.350*	TGATGGCAAATGAGGTCAAA	CAGCATAACAATTGGCTTGG
NPF	*chr2.CM0826.370*	GCTGTGCTGGGAGTACTGAA	TGCTTCTTGCACACCAATGT
NPF	*chr4.CM0170.40*	GGCCCAAGAATGAAATCTGA	TGACGGTTCCCTTTCCTAAA
NPF	*chr4.CM0247.130*	GCTTACATTGCAGTCGCAAA	CGGGTAAAAAGGGCTTCTCT
NPF	*chr4.LjB20H09.30*	AAGGACAAAAGGCTTGGCTCA	TGATCGATTTTTCGGGACTAA
NPF	*chr6.CM0118.580*	AGGCCTCAAGCCCTTACAGT	TCCCCAAATTGTCTTATTTTTCA
LjNPF1.7	*LjSGA_046844.2*	ACCATGAGACGAAATGTCGCC	CGAGCAAGACAAACTCCGGAA
NRT2	*chr1.CM0001.20*	GAGGCATGTTTTGTGGTCCT	GCCATACAGGGTATAGGTTGG

LjNRT2.1	*chr3.CM0649.40*	CTGCTCCAACTCCTCCAAAC	AGACCCACACTCCCACAGAC
LjNRT2.2	*chr3.CM0649.30*	TAATGGATTCCCTGGCTGAA	ATGGCAAAACCAACATGTGA
NRT2	*chr4.CM0161.180*	GGAGCAGCAGAAAGGATCAC	GGTTGTGCTACAAGGGAGGA
LjCLCa	*chr1.CM0064.1040.r2.m*	ACAAGATCAAATGGCGCTGG	GCGAAACGCTGCACAAACT
LjN70	*LjSGA_018310.1*	ATTCCCAACTGTGGCACATGT	TCAAATACCCCTCTGCAATGC
LjLBD38	*chr4.CM0128.420.r2.m*	AACGACCCGCTCTGTTTCAGTC	TTGACCGGGTTCACCGTTCTTC
LjCIPK8	*LjSGA_048938.1*	TGATCAAGCTCTTGCGGCTGTG	AGCTCGAAGGCTTTCAGTCGTG
LjCIPK23	*chr6.CM0037.1410.r2.m*	CAGAGTATCTCCTTCAGACTTGCG	TTGGAGGTGGCTCCTTCACTTC
LjANR1	*chr6.CM0055.400.r2.m*	AAGCCAGCCACAGCCTCAATTC	CAGCTGTAATCCCAGTTTCACTGC
LjCLE-RS2	*chr3.CM2103.25.r2.a*	GCTCGTAATCTCCAAATCATTCACA	GGTGAGAGTCTTTGCTGTTGATATCC
LjNLP7/NIN	*chr5.CM0148.170.r2.a*	AATGAAGCGCATCTGTAGGCA	TGGACGGATTCAATAACACGC

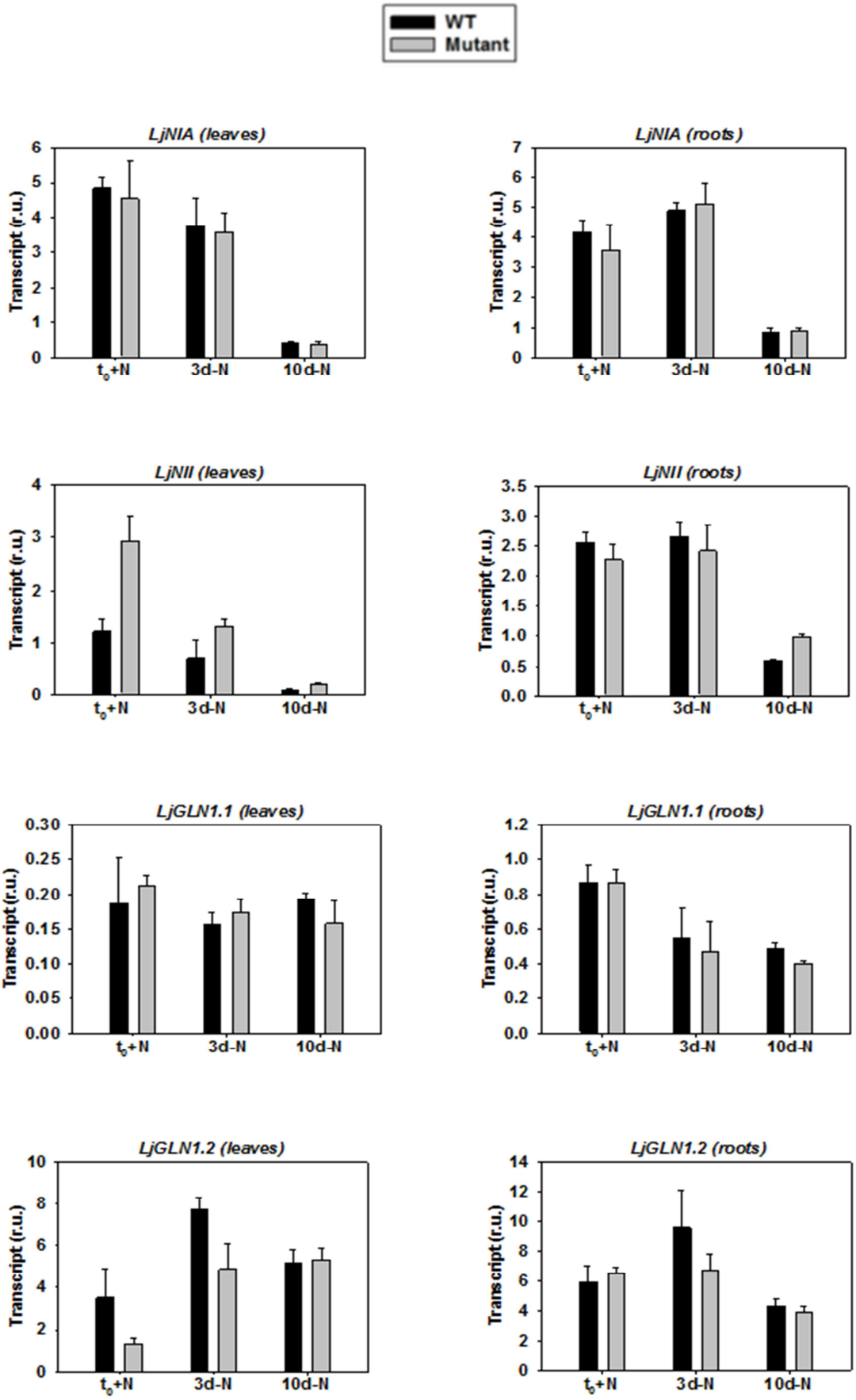
Supplemental Table S1. List of oligonucleotides used for qRT-PCR measurements. Sequence data and codes for each gene can be found in the Kazusa 2.5 database (http://www.kazusa.or.jp/lotus). Other details as described in legend for Table 2.

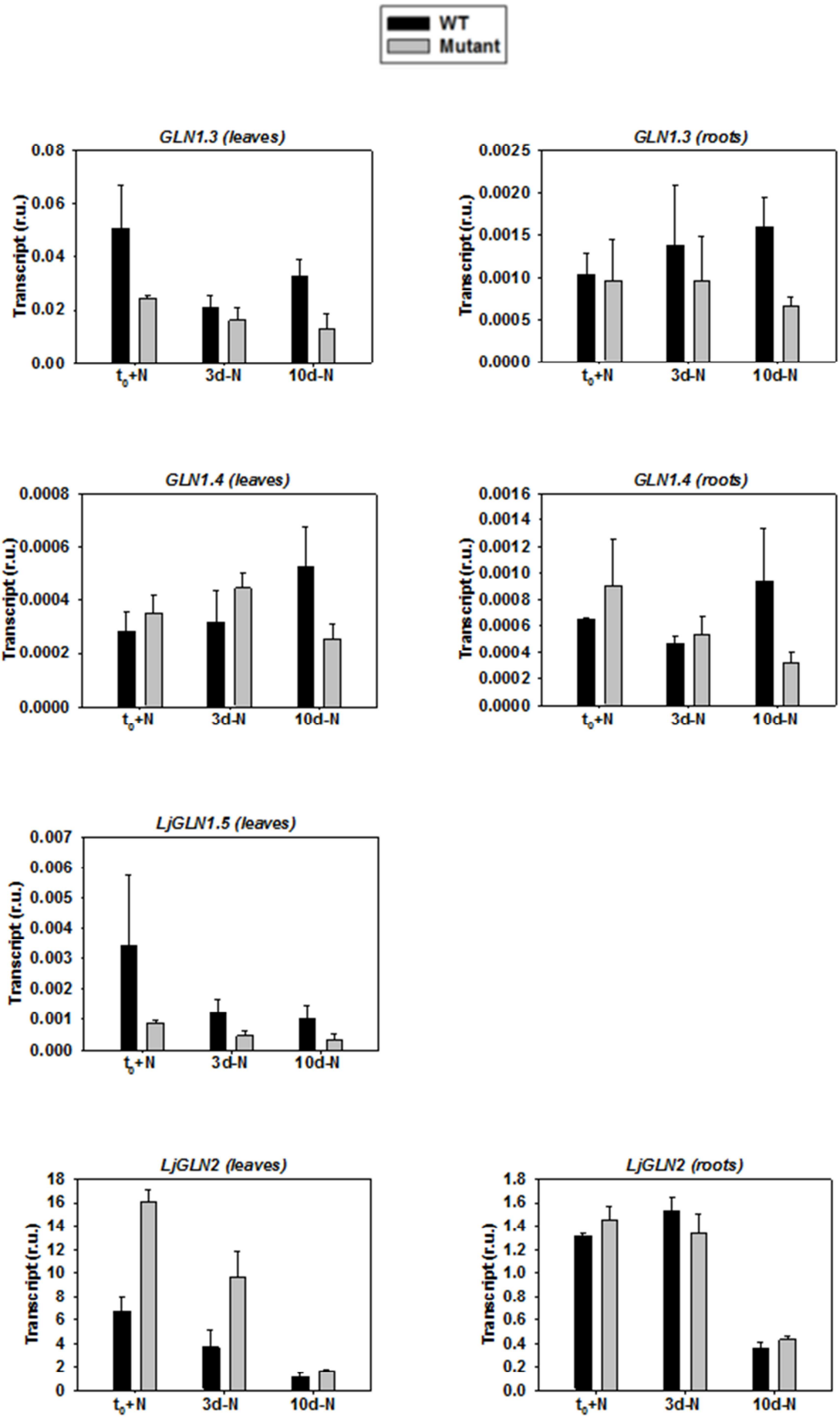
Supplemental Fig. S1

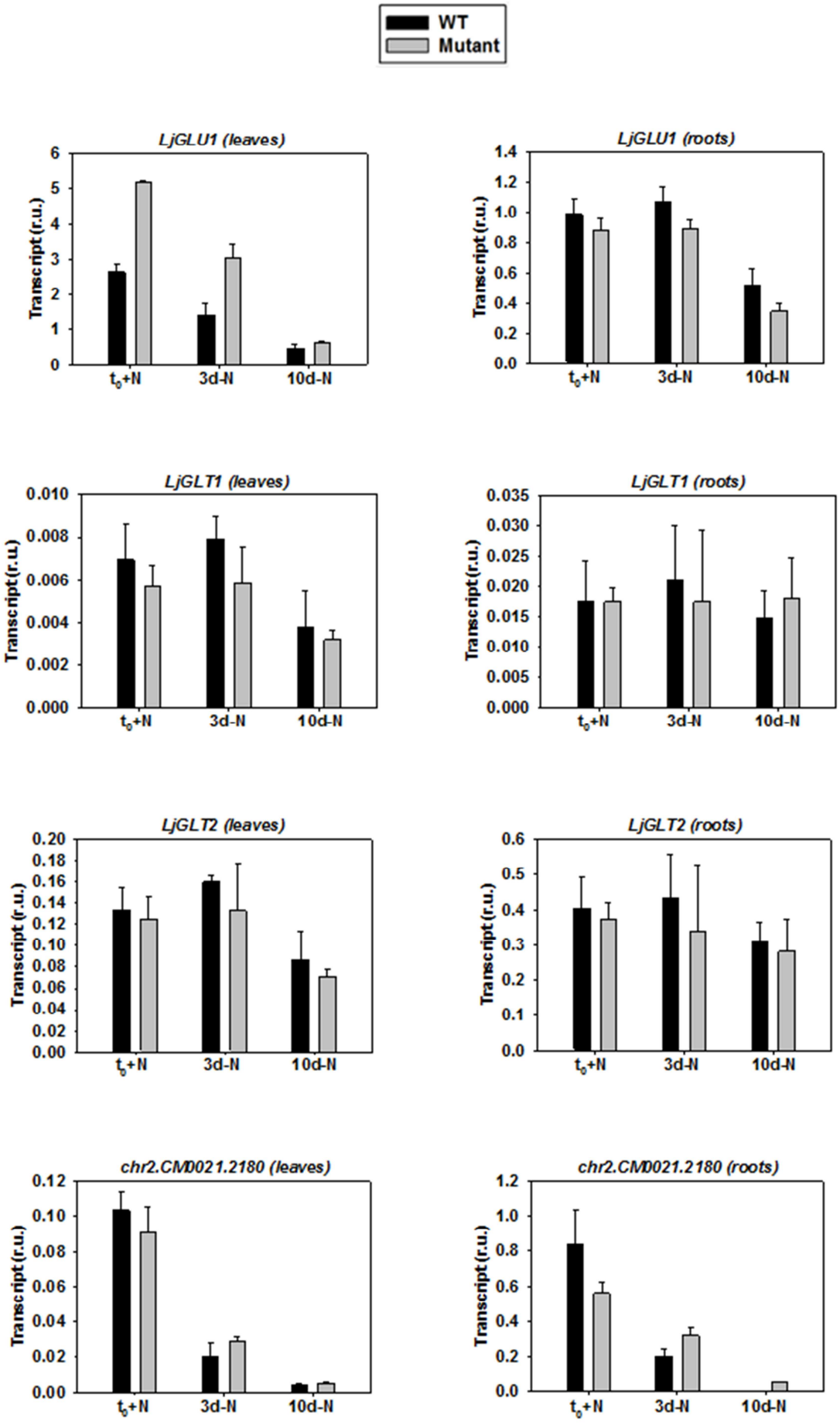


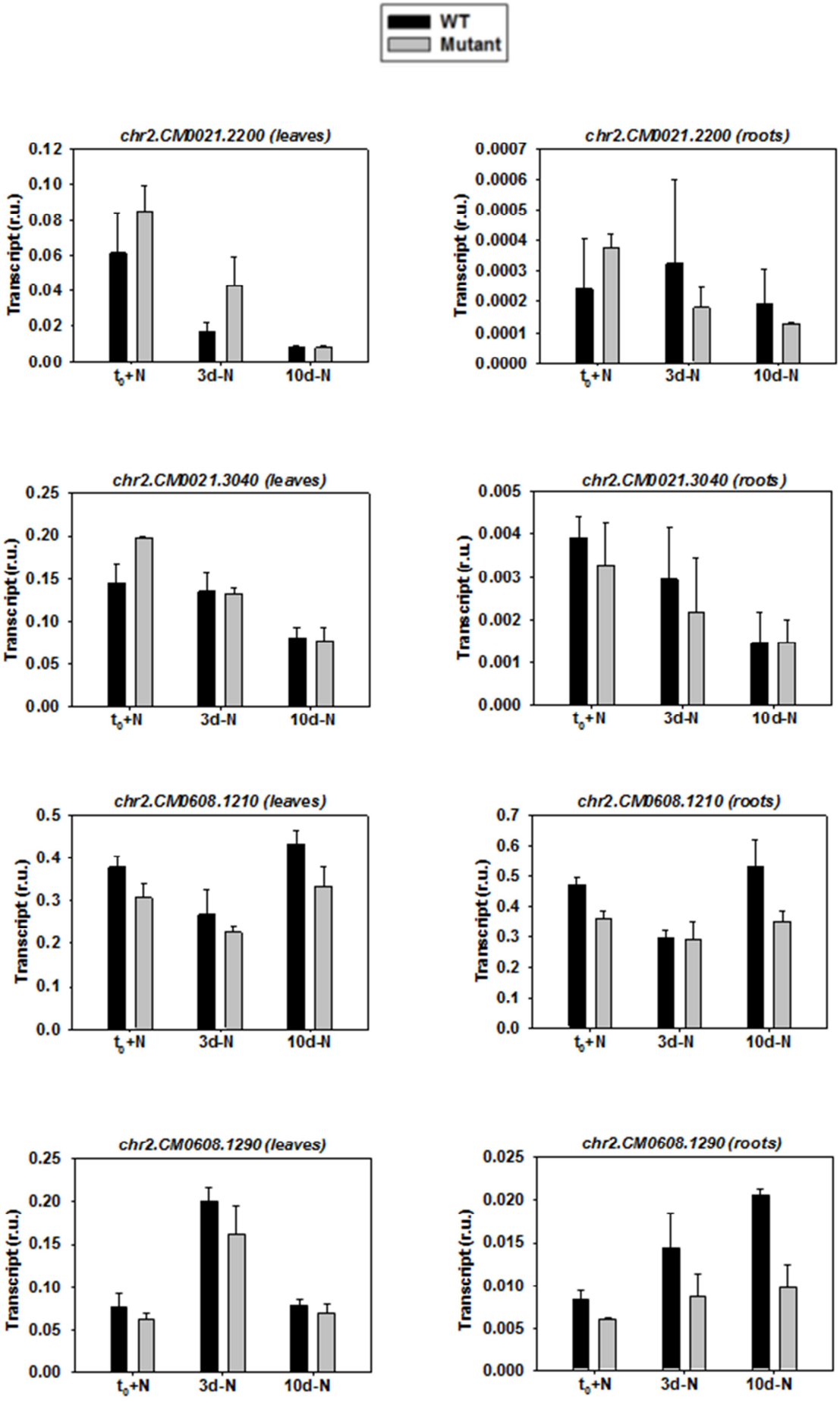
Supplemental Fig. S1. Analysis of the back cross progeny of *Ljclo1* (♀) x WT (♂). The figure shows the measurements of membrane potential (ΔE_m) for nitrate uptake electrophysiological measurements (A), as well as LATS nitrate uptake by nitrate depletion (B) and shoot-tip chlorophyll content (C) for each of 16 independents F2 plants from backcross progeny, together with WT and parental *Ljclo1* controls. D and E panels show average plant growth parameters determined in F2 plants showing either WT (b1-b13) or *Ljclo1* mutant (b14-b16) phenotypes together with parental lines.

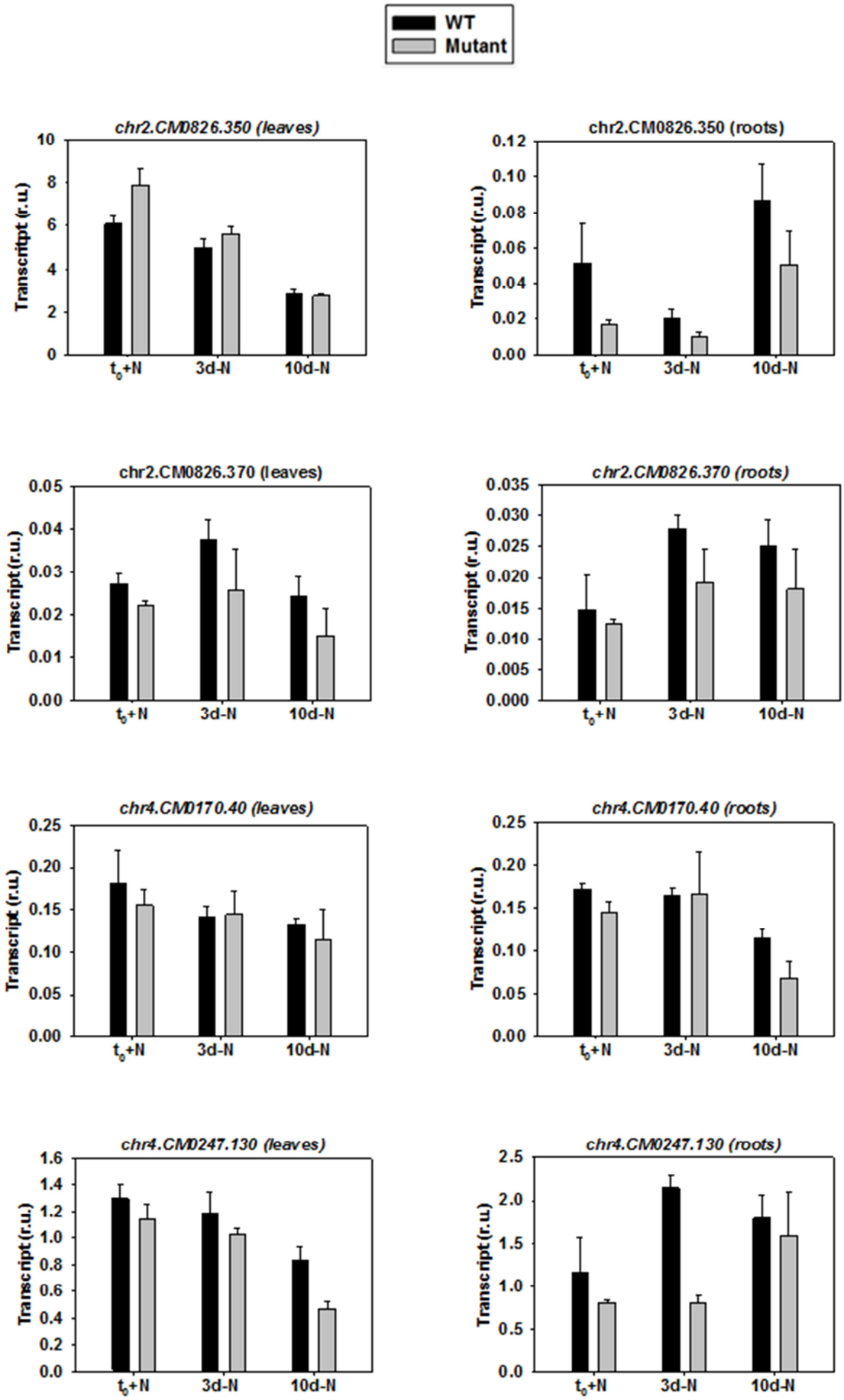
Supplemental Fig. S2

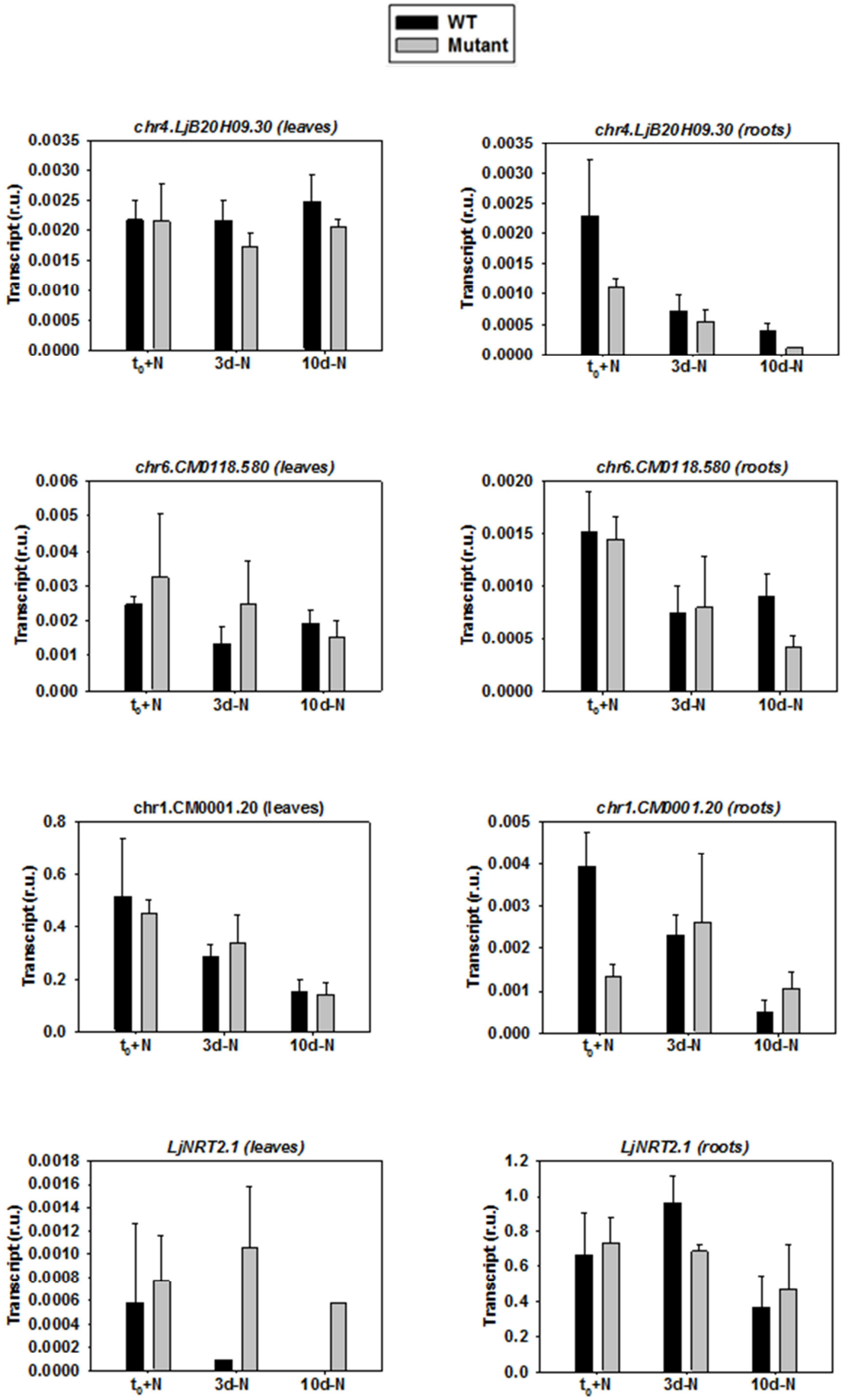


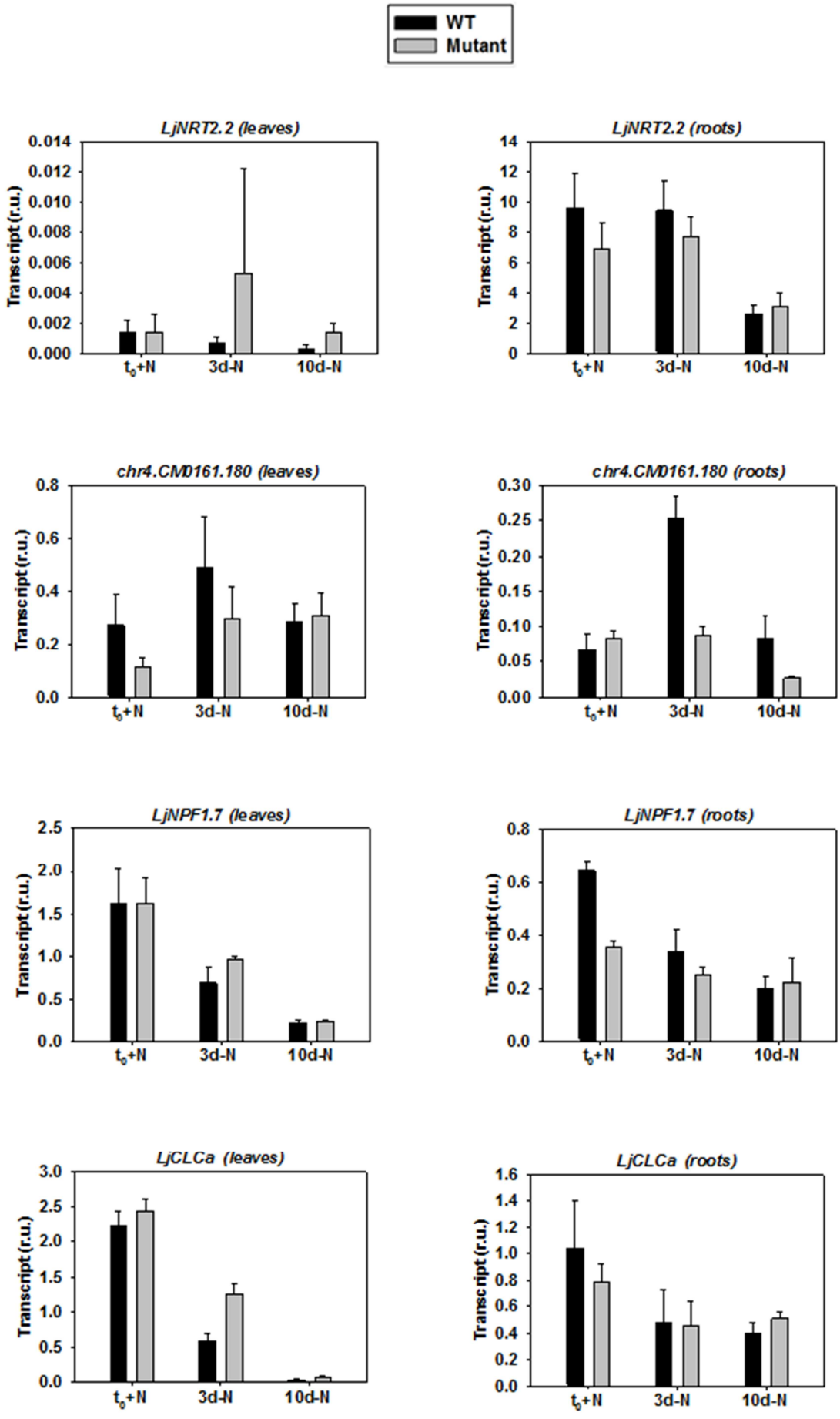

WT
Mutant
GLN1.3 (leaves)
Transcript (r.u.)
0.08
0.06
0.04
0.02
0.00
t0+N
3d-N
10d-N
GLN1.3 (roots)
Transcript (r.u.)
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
t0+N
3d-N
10d-N
GLN1.4 (leaves)
Transcript (r.u.)
0.0008
0.0006
0.0004
0.0002
0.0000
t0+N
3d-N
10d-N
GLN1.4 (roots)
Transcript (r.u.)
0.0016
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
t0+N
3d-N
10d-N
LjGLN1.5 (leaves)
Transcript (r.u.)
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
t0+N
3d-N
10d-N
LjGLN2 (leaves)
Transcript (r.u.)
18
16
14
12
10
8
6
4
2
0
t0+N
3d-N
10d-N
LjGLN2 (roots)
Transcript (r.u.)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
t0+N
3d-N
10d-N

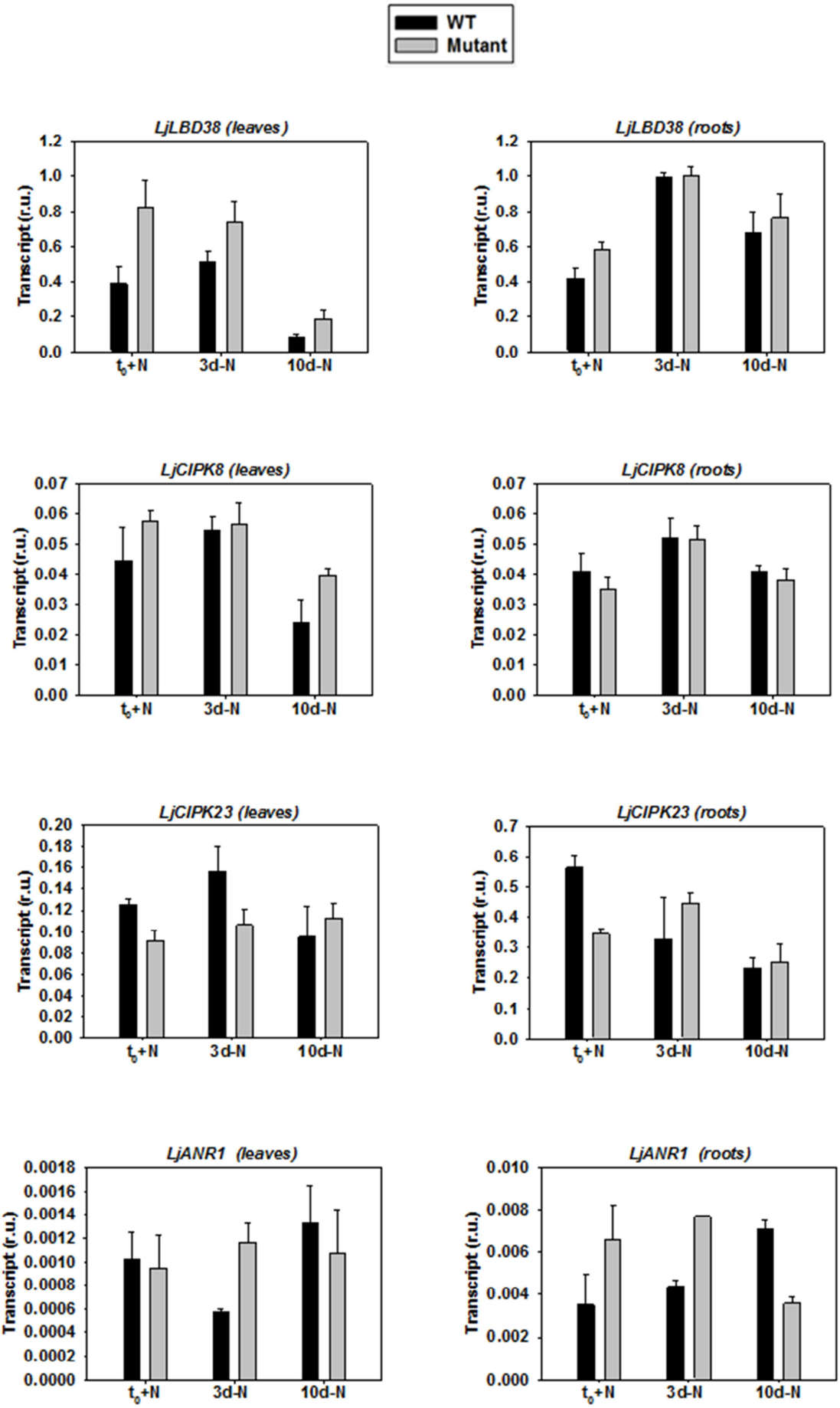

WT
Mutant
LjGLU1 (leaves)
LjGLU1 (roots)
LjGLT1 (leaves)
LjGLT1 (roots)
LjGLT2 (leaves)
LjGLT2 (roots)
chr2.CM0021.2180 (leaves)
chr2.CM0021.2180 (roots)
Transcript (r.u.)
t0+N
3d-N
10d-N

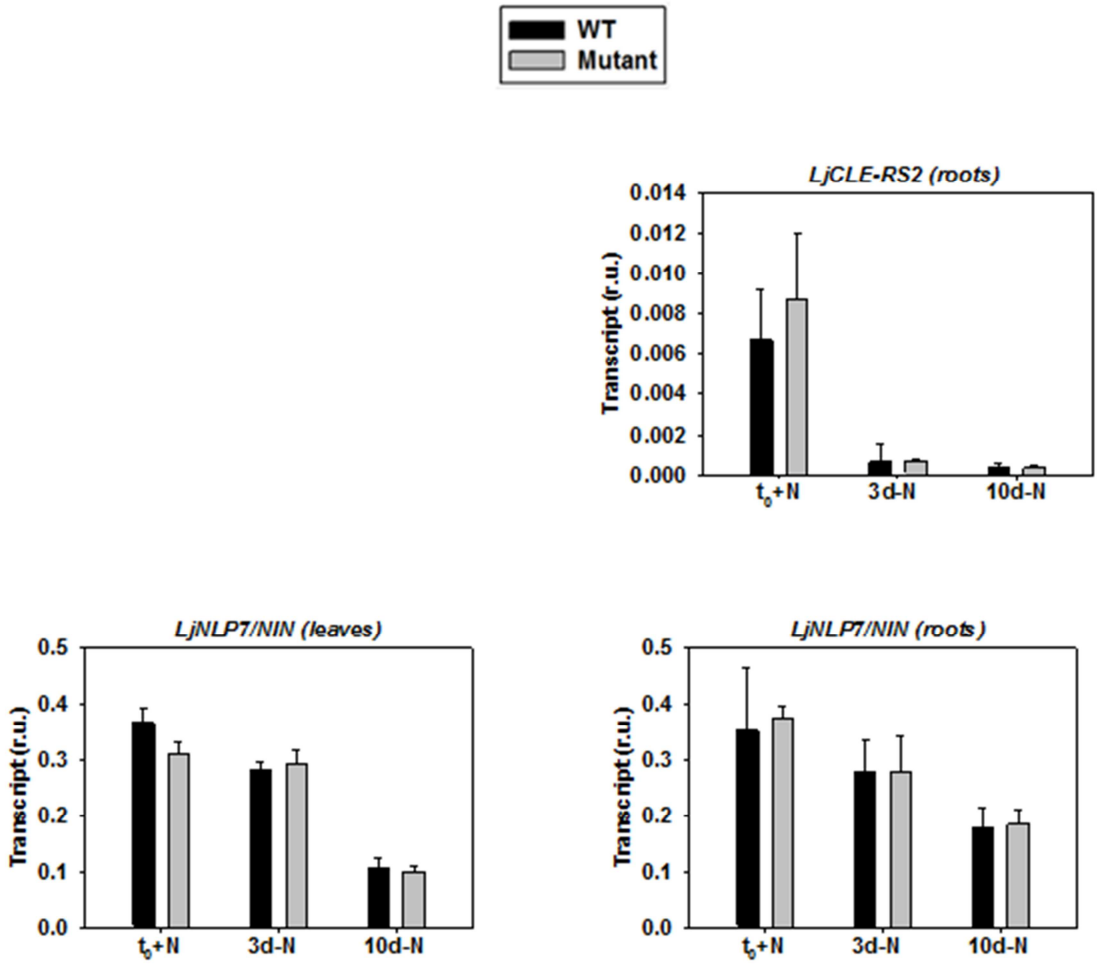

WT
Mutant
chr2.CM0021.2200 (leaves)
chr2.CM0021.2200 (roots)
chr2.CM0021.3040 (leaves)
chr2.CM0021.3040 (roots)
chr2.CM0608.1210 (leaves)
chr2.CM0608.1210 (roots)
chr2.CM0608.1290 (leaves)
chr2.CM0608.1290 (roots)
Transcript (r.u.)
t0+N
3d-N
10d-N


WT
Mutant
chr2.CM0826.350 (leaves)
chr2.CM0826.350 (roots)
chr2.CM0826.370 (leaves)
chr2.CM0826.370 (roots)
chr4.CM0170.40 (leaves)
chr4.CM0170.40 (roots)
chr4.CM0247.130 (leaves)
chr4.CM0247.130 (roots)
Transcript (r.u.)
t0+N
3d-N
10d-N


WT
Mutant
chr4.LjB20H09.30 (leaves)
chr4.LjB20H09.30 (roots)
chr6.CM0118.580 (leaves)
chr6.CM0118.580 (roots)
chr1.CM0001.20 (leaves)
chr1.CM0001.20 (roots)
LjNRT2.1 (leaves)
LjNRT2.1 (roots)
Transcript (r.u.)
t0+N
3d-N
10d-N


WT
Mutant
LjNRT2.2 (leaves)
LjNRT2.2 (roots)
chr4.CM0161.180 (leaves)
chr4.CM0161.180 (roots)
LjNPF1.7 (leaves)
LjNPF1.7 (roots)
LjCLCa (leaves)
LjCLCa (roots)
Transcript (r.u.)
t0+N
3d-N
10d-N


WT
Mutant
LjLBD38 (leaves)
LjLBD38 (roots)
LjCIPK8 (leaves)
LjCIPK8 (roots)
LjCIPK23 (leaves)
LjCIPK23 (roots)
LjANR1 (leaves)
LjANR1 (roots)
Transcript (r.u.)
t0+N
3d-N
10d-N



Supplemental Fig. S2. qRT-PCR measurements of the transcript levels of nitrate-related genes in WT and *Ljclo1* mutant plants after 0, 3 and 10 days of transfer from standard Hornum medium to N-free medium (N-starvation conditions) in hydroponic cultures (t_0+N, 3d-N and 10d-N, respectively). Genes analyzed were the same as listed in Table 2. The lack of one particular plot means undetectable levels of transcripts for that particular gene or tissue.

DISCUSIÓN

La realización de esta Tesis Doctoral perseguía como objetivo principal el estudio del metabolismo del nitrógeno en relación con la fotorrespiración y el estrés abiótico empleando herramientas de genómica funcional. Tanto la asimilación de nitrógeno como el estrés abiótico, particularmente la sequía, son dos de los factores que más limitan el crecimiento de las plantas en el género *Lotus* y de ahí el interés de utilizar la leguminosa modelo *Lotus japonicus* para este trabajo, ya que para esta planta se han desarrollado recientemente numerosas herramientas de genómica funcional (LOTASSA, 2010; Márquez, 2005)

La posibilidad de contar en nuestro laboratorio con mutantes fotorrespiratorios deficientes en GS2 de plantas de *L. japonicus*, facilitó el estudio de la fotorrespiración, desde el punto de vista de su interconexión con el metabolismo del nitrógeno, y de la relación a su vez del metabolismo del nitrógeno con el estrés abiótico.

1. Reasimilación de amonio fotorrespiratorio.

Una de las características principales descrita en plantas mutantes fotorrespiratorias de cebada deficientes en GS2, es la gran acumulación de amonio que experimentan cuando son transferidas desde la atmósfera de alto CO_2 (fotorrespiración suprimida) a una atmósfera de aire donde comienzan a fotorrespirar (Wallsgrove *et al.*, 1987; Blackwell *et al.*, 1988). En base a estos resultados se sugirió por primera vez que era la GS2, y no la GS1, la enzima responsable de la asimilación del amonio producido durante la fotorrespiración.

En esta Tesis se han llevado a cabo nuevos estudios con mutantes deficientes en GS2 de la leguminosa modelo *L. japonicus* que es la segunda especie vegetal y primera leguminosa donde se han descrito estos mutantes (cf. introducción). Para los mutantes *Ljgln2-2* de *Lotus japonicus*, al igual que para los mutantes deficientes en GS2 de cebada, también se observó una elevada acumulación de amonio cuando las plantas se transferían desde una atmósfera de alto CO_2 a una atmósfera de aire, donde la fotorrespiración está activa. Sin embargo, se detectó que los niveles de amonio alcanzaban un máximo a los tres días de dicha transferencia, tras el cual se daba una disminución progresiva de los mismos hasta estabilizarse a los ocho días de la transferencia a aire.

Esta caída de los niveles de amonio sugería que las plantas mutantes estaban poniendo en marcha mecanismos de destoxificación para disminuir los niveles de este metabolito. Se estudió más en detalle esta caída de los niveles de amonio y se postuló que podía ser debida a dos efectos. Por un lado, se demostró que se producía una represión coordinada de la transcripción de los genes fotorrespiratorios en las plantas mutantes, de manera que los niveles de transcrito

de estos genes tenían una tendencia opuesta a los niveles de amonio en dicho genotipo al transferirlas plantas a condiciones donde la fotorrespiración está activa. Este "apagado" coordinado de la fotorrespiración contribuiría a que no se produjese más amonio fotorrespiratorio en las plantas deficientes en GS2 (publicación 1). Por otro lado, se ha demostrado que existe una inducción de rutas alternativas que podían contribuir a que descendiesen los niveles de amonio en las plantas mutantes. Se comprobó que había una inducción de glutamina sintetasa citosólica (GS1), glutamato deshidrogenasa (GDH) y aparragina sintetasa (AS) que podían favorecer esta disminución de los niveles de amonio (publicación 2), lo que implicó a su vez un análisis exhaustivo de todos los genes del genoma de *L. japonicus* que codifican para éstas y otras enzimas implicadas en la asimilación de amonio (Publicación 8).

Estos resultados son particularmente interesantes pues existen otros estudios que han descrito que existe una inducción de GS1, ASN y GDH en hojas senescentes para reasimilar el amonio producido por la degradación de proteínas (Masclaux-Daubresse *et al.*, 2010). Nuestros resultados indican que es posible que los procesos de destoxificación de amonio fotorrespiratorio y reasimilación de amonio resultante de degradación proteica puedan compartir mecanismos similares.

En cuanto a la glutamina sintetasa, varios estudios de localización tisular de las diferentes isoenzimas de GS (Edwards *et al.*, 1990; Tobin y Yamaya, 2001; Teixeira *et al*., 2005) han revelado que las GS1 tienden a estar presentes en tipos celulares distintos a los de las GS2. Mientras que las primeras se encuentran en células del tejido vascular, las segundas son características de tejidos fotosintéticos como las células del mesófilo. Esta circunstancia explicaría presumiblemente que las GS1 no puedan compensar completamente la deficiencia en GS2 en los mutantes fotorrespiratorios por el hecho de expresarse en tipos celulares diferentes. A pesar de ello, los estudios abordados en *L. japonicus* indican que la GS1 sí puede contribuir significativamente a disminuir los niveles de amonio fotorrespiratorio que se acumula en la planta.

Por otro lado, se han descrito altos niveles de NH_4^+ y glutamina en plantas transformadas de tabaco deficientes en la actividad Fd-GOGAT transferidas a aire. Dado que estas plantas poseían niveles estables de glutamato, se sugería la existencia de una ruta alternativa para la síntesis de este aminoácido en estas plantas transgénicas con baja actividad Fd-GOGAT. Los autores pensaron que esta ruta podría ser la NAD(H)-GDH ya que mostraba una actividad elevada en las plantas transformadas. De esta forma, se asignó un papel a la NAD(H)-GDH en la síntesis compensatoria de glutamato cuando la actividad de la Fd-GOGAT se encuentra disminuida y el amonio actuaría como señal responsable de la

inducción de la actividad de la enzima (Ferrario-Mery *et al*., 2002). Sin embargo, evidencias recientes sugieren que la GDH trabaja en la dirección de la desaminación de glutamato para producir 2-oxoglutarato, cuyo destino será el ciclo de Krebs (Fontaine *et al*., 2012; Labboun *et al.,* 2009). Teniendo en cuenta los altos niveles de NH_4^+ presentes en las hojas de *Ljgln2-2*, una posibilidad es que se dé un incremento de la desaminación de glutamato para proveer de esqueletos carbonados para su reasimilación.

En cuanto a la asparragina sintetasa, se sabe que el sustrato donador de grupos amino para la reacción catalizada por esta enzima es la glutamina. Sin embargo, está descrito que esta enzima puede usar también NH_4^+ para la síntesis de asparragina siempre que la concentración de éste sea lo suficientemente grande (Lea *et al*., 2007; Coruzzi *et al*., 2000). Este hecho se ha descrito especialmente en situaciones de estrés, donde la ASN facilitaría la destoxificación de amonio (Wong *et al*., 2004). Además, un estudio en hojas de tabaco ha demostrado que el $^{15}NH_4^+$ se incorpora a asparragina por la acción de la ASN (Masclaux-Daubresse *et al*., 2006).

La toxicidad por amonio y los posibles mecanismos de destoxificación subyacentes a la misma, constituyen un tema de bastante interés en la bioquímica de la nutrición nitrogenada en las plantas. Se han descrito un gran número de explicaciones sobre los posibles mecanismos de toxicidad del NH_4^+, aunque en muchos casos algunas de las teorías existentes aportan explicaciones insuficientes, parciales o incorrectas (Britto *et al*., 2002). Entre éstas se encuentran el efecto desacoplante del NH_4^+ en la fotofosforilación; el efecto de la disminución del pH externo o balance del pH interno como resultado de la adquisición de NH_4^+; la acumulación *per se* de NH_4^+ libre en tejidos de la planta (incluyendo, expresamente, el citosol); y la necesidad de una mayor demanda de carbono de la raíz para la síntesis de aminoácidos bajo nutrición con NH_4^+. Explicaciones más plausibles y que requieren aún más investigación incluyen la participación de la síntesis y acción del etileno en la respuesta de la planta a la situación de estrés producida por NH_4^+; los procesos de flujo a través de la membrana del NH_4^+, y, en particular, la salida ("efflux") del mismo que puede ser demandante de energía; efectos fotosintéticos, relacionados particularmente con la fotoprotección; y la alteración de las concentraciones de posibles cationes esenciales de sus niveles homeostáticos en distintos compartimentos subcelulares. Además, se podría aprender mucho sobre la toxicidad del amonio examinando el alivio de su toxicidad, particularmente a través de la co-presencia de nitrato (Britto *et al.,* 2002). De hecho, en la publicación 3 de la Tesis se muestran interesantes diferencias en la expresión de los genes entre las plantas cultivadas exclusivamente con amonio o con nitrato amónico, siendo esta última

la fuente nitrogenada óptima para el cultivo de la mayoría de las plantas, incluida *Lotus japonicus* (Márquez, 2005).

En el caso de mutantes fotorrespiratorios deficientes en la asimilación de amonio en cebada se ha postulado que las deficiencias de crecimiento en condiciones de fotorrespiración activa pueden ser debidas a una deficiencia de nitrógeno más que a una acumulación de amonio *per se* (Häuser *et al*., 1994; Joy et *al.*, 1992). Sin embargo, los estudios abordados con los mutantes *Ljgln2-2* parecen excluir la posibilidad de una deficiencia de nitrógeno en estas plantas ya que, por el contrario, con la transferencia a condiciones de fotorrespiración activa se incrementaban los niveles de varios aminoácidos en este genotipo (publicación 1 y 2), en contra de lo descrito en cebada. Por este motivo, los resultados mostrados en esta Tesis Doctoral sugieren que la respuesta de las plantas mutantes a condiciones de fotorrespiración activa está determinada, directa o indirectamente, por el NH_4^+ fotorrespiratorio acumulado en las hojas. No obstante, no podemos decir con claridad si este efecto se produce sólo por el amonio fotorrespiratorio *per se* o si es general para todas las situaciones en las que hay alta concentración de amonio en hojas de *L. japonicus*. Otra serie de experimentos serían necesarios para examinar en mayor profundidad esta cuestión como los que se citan a continuación: a) una posibilidad sería el estudio de plantas silvestres tratadas con un un inhibidor de glutamina sintetasa como la metionina sulfoximina (MSX) o la fosfinotricina (PPT); en nuestro laboratorio hemos comprobado que plantas de *L. japonicus* sometidas a estos tratamientos incrementan notablemente su contenido en NH_4^+, tal y como se ha descrito en otras plantas (Walker *et al*., 1984; Husted y Schjoerring, 1995), pero esto es debido a una inhibición tanto de la GS1 como de la GS2 con lo que no sería un modelo comparable a la situación de los mutantes fotorrespiratorios examinados que sólo están afectados en GS2; b) otra posibilidad sería el estudio de plantas cultivadas con NH_4^+ como fuente de nitrógeno pero se ha comprobado que estas plantas no acumulaban amonio en hojas, ya que *Lotus japonicus* asimila el nitrógeno en raíces y lo transporta a las hojas, mayoritariamente, en forma de asparragina, con lo cual tampoco son de utilidad para la comparación con los mutantes fotorrespiratorios deficientes en GS2 que acumulan altas cantidades de amonio en hojas; y c) una tercera posibilidad sería el cultivo de plantas *Ljgln2-2* en atmósfera de alto CO_2 y la pulverización de las hojas con un spray que contenga NH_4^+ de forma que éste pueda penetrar y acumularse en las hojas en condiciones de fotorrespiración suprimida hasta niveles comparables con los generados en el mutante *Ljgln2-2* en condiciones de fotorrespiración activa. Aún no ha podido comprobarse si con este procedimiento se daría el resultado esperado dada la posible toxicidad del amonio para las hojas siendo, en todo

caso, necesario encontrar la concentración externa adecuada del mismo, lo que requeriría largo tiempo de puesta a punto.

Es interesante mencionar que los mutantes de GS de cebada también mostraban una alta acumulación de amonio, sin embargo, presentaban una curva de acumulación gradual de este compuesto hasta alcanzar un nivel máximo de saturación posiblemente provocada por el equilibrio entre la producción interna de amonio y la difusión de éste al exterior por los estomas de las hojas (Blackwell *et al*., 1987). Sin embargo, estos estudios no mostraron ninguna disminución posterior de los niveles de amonio, como la observada para el mutante *Ljgln2-2* estudiado en esta Tesis. No obstante, el estudio realizado en plantas de cebada se realizó en intervalos de transferencia a aire muy cortos comparados con los nuestros, por lo que no es posible saber con certeza si el comportamiento y los mecanismos de posible destoxificación de amonio observados en los mutantes deficientes en GS2 de *L. japonicus* podrían darse también en otras plantas.

2. Interacción de la GS2 con el metabolismo del carbono y del nitrógeno.

La publicación 1 muestra que hay una gran modulación del transcriptoma de plantas silvestres y *Ljgln2-2* cuando éstas se transfieren desde una atmósfera de alto CO_2 a una atmósfera de niveles normales de CO_2. Esta modulación se da en mucho mayor grado en las plantas mutantes, en las que se detectan más de 120 familias génicas con genes modulados específicamente en el mutante *Ljgln2-2* y no en las plantas silvestres. La mayoría de dichos genes pertenecen al metabolismo primario y secundario, incluyendo, por ejemplo, la biosíntesis de clorofila, el metabolismo de aminoácidos, la glucolisis, el ciclo de Krebs y el metabolismo de la sacarosa y el almidón u otros aspectos del metabolismo de carbono, entre otros. En esta publicación se muestra claramente que la fotorrespiración está relacionada con muchas rutas metabólicas y que la deficiencia en GS2 afecta en gran medida a la asimilación de carbono en plantas de *Lotus japonicus*. En este sentido, los datos presentados en esta Tesis concuerdan a su vez con otros estudios recientemente obtenidos en nuestro laboratorio. En el trabajo de García-Calderón *et al.* (2012) se describe que la transferencia de plantas silvestres y *Ljgln2-2* desde una atmósfera enriquecida en alto CO_2 a aire afecta al proceso de nodulación, especialmente en los mutantes deficientes en GS2, debido a una reducción de la disponibilidad de carbono, especialmente en *Ljgln2-2*. De hecho, incluso en atmósfera de alto CO_2, en los mutantes deficientes en GS2 se detectan niveles menores de almidón, sacarosa, glucosa y fructosa que en las plantas silvestres, confirmando así la implicación de la GS2 en el balance de carbono de la planta

Por otro lado, en la publicación 3 se evidencia que hay un gran número de genes que se modulan en las hojas de *L. japonicus* tanto por la deficiencia de GS2 en condiciones de fotorrespiración suprimida como por la disminución de la concentración de CO_2 atmosférico en las plantas silvestres. Además, los genes modulados en ambas condiciones tienen una magnitud de cambio muy similar. Entre estos genes se encuentran varios que están directamente relacionados con el metabolismo del carbono.

También se ha demostrado que la deficiencia en GS2 provocaba una respuesta diferente u opuesta en hojas de plantas cultivadas con nutrición mixta que en hojas de plantas cultivadas con nitrato o con amonio como fuentes nitrogenadas (publicaciones 3 y 6). Cabe destacar, asimismo, que la comparación de los transcriptomas de hojas cultivadas en nutrición mixta con con los de hojas de plantas noduladas, muestra que hay una expresión diferencial de genes del metabolismo de carbono en estas dos condiciones. Este comportamiento no lo adoptaban ni las plantas cultivadas únicamente con nitrato ni con amonio en comparación con las plantas noduladas.

Todas estas evidencias ponen de manifiesto cómo un defecto en la asimilación de nitrógeno afecta al metabolismo del carbono en *Lotus japonicus*. Es, pues, probable, que la GS2 tenga un papel crucial en la señalización del balance C/N en las plantas de *L. japonicus*.

En otras publicaciones de la Tesis se ha hecho además un estudio exhaustivo sobre los niveles de expresión de genes implicados en la utilización de nitrato o amonio en las plantas de *L. japonicus.* Por ejemplo, en la publicación 9 se ilustran los niveles de expresión de 35 genes implicados en el transporte, reducción o regulación de nitrato en las plantas silvestres de *L. japonicus,* en comparación con los datos obtenidos para el mutante *Ljclo1* afectado en el consumo de nitrato. Igualmente, la publicación 8 recoge los niveles de expresión de los distintos genes implicados en la asimilación de amonio para distintos órganos de las plantas de *L. japonicus*.

3. Interacción entre el metabolismo del nitrógeno y el estrés abiótico.

En las publicaciones 4 y 5 se muestran los efectos que produce la sequía en el transcriptoma de las hojas de *L. japonicus*. Cuando dichas plantas se sometían a sequía se modulaban varias rutas metabólicas, entre las que se incluían el metabolismo del almidón y la sacarosa, el metabolismo de aminoácidos y el metabolismo de clorofila y porfirina. Las plantas de *L. japonicus* sometidas a estrés hídrico experimentaban una represión de los genes de la fotosíntesis. Esta respuesta se ha observado en otras plantas (Saibo *et al.*,

2009). El metabolismo central del carbono se afectaba con una represión general de los genes del ciclo de Krebs. Considerando que los bajos niveles de fotosíntesis darán lugar a una reducida disponibilidad de energía y de carbono, es fácilmente explicable la modulación del metabolismo del almidón y sacarosa, cuya finalidad es movilizar las reservas de carbono. Además, los cambios transcriptómicos asociados con el metabolismo de aminoácidos podría ser otro modo de abastecer el ciclo de Krebs bajo condiciones de sequía (publicación 5). Estos estudios y otros recientes (Yousfi *et al*., 2012) han mostrado una interconexión entre el metabolismo de nitrógeno y la adaptación de las plantas al estrés hídrico.

Además, en las publicaciones 4 y 5 de la Tesis se ha mostrado también un estudio comparado sobre el efecto de la sequía en el transcriptoma de hojas de plantas silvestres y mutantes deficientes en GS2 de *L. japonicus*. El mayor número de genes modulados por sequía exclusivamente en los mutantes deficientes en GS2, así como la intensidad en la modulación de los mismos., comparadas con las del organismo silvestre en las mismas condiciones, confirma la clara interconexión existente entre el estrés hídrico y el metabolismo del nitrógeno en las plantas de *L. japonicus*. Además, las rutas metabólicas más moduladas de manera específica en los mutantes deficientes en GS2 coincidían en gran parte con las que eran específicamente moduladas en dichos mutantes en condiciones de fotorrespiración activa y sin sequía, anteriormente mencionadas. Así, las principales rutas moduladas por sequía en los mutantes deficientes en GS2 incluían el metabolismo de la porfirina y la clorofila, el metabolismo del almidón y sacarosa, el metabolismo de aminoácidos y el metabolismo de los alcaloides. Otros trabajos anteriores demostraron una clara implicación de la GS2 en la síntesis de prolina (Díaz *et al.,* 2010). La prolina es un aminoácido producido por las plantas como respuesta a diferentes situaciones de estrés abiótico (Szabados & Savoure, 2010). En el caso de las plantas del género *Lotus*, podemos decir que la prolina participa en distintas respuestas a estrés que incluyen no solamente, como el estrés por aluminio (publicación 5).

Todas estas evidencias ponen de manifiesto que existe una interconexión entre el metabolismo del nitrógeno y el estrés abiótico, particularmente el estrés hídrico, y que la GS2 está relacionada con ambos procesos en *L. japonicus*.

4. Visión global y nuevas perspectivas.

En esta Tesis Doctoral se han obtenido resultados que ponen de manifiesto la existencia de una interesante relación entre el balance C/N de las plantas de *L. japonicus* con el metabolismo fotorrespiratorio (publicaciones 1, 2 y 3) y con el estrés abiótico, concretamente, con el estrés hídrico (publicaciones 4 y 5)

señalándose un papel fundamental de la GS2 en ambos procesos (Figura 1). Las publicaciones 6, 7, 8 y 9 también aportan valiosa información sobre estos tópicos.

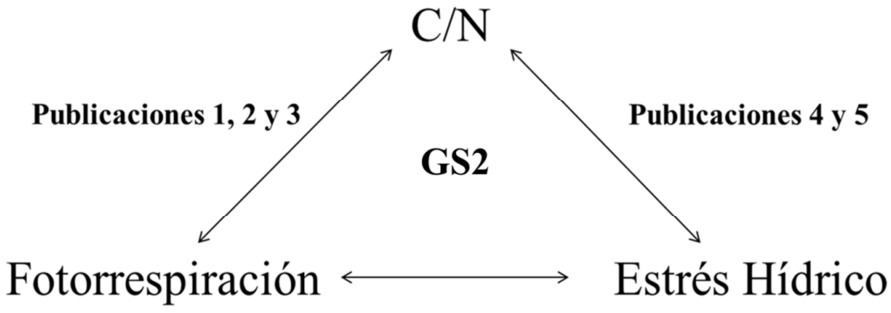


Figura 1. Tópicos de estudio de esta Tesis Doctoral.

Por último, cabe destacar también que en los trabajos que componen esta Tesis Doctoral se describen varios factores de transcripción que pueden tener papeles clave en la respuesta a las condiciones de fotorrespiración activa, a la presencia / ausencia de nitrógeno externo, a las diferentes fuentes nitrogenadas minerales, a los cambios en la relación C/N o a la sequía. Actualmente, se dispone de una colección de mutantes por transposones LORE1 (Urbanski *et al*., 2012) que puede ser de gran utilidad para el esclarecimiento de las funciones de dichos factores de transcripción en estudios futuros. Además, se está colaborando con el grupo de investigación del Dr. Rodrigo Gutiérrez de la Pontificia Universidad Católica de Chile para poner a punto dos herramientas bioinformáticas que permitirán establecer predicciones de genes relacionados con un conjunto de genes de interés en *L. japonicus*. Dichas herramientas facilitarán el estudio de estos factores de transcripción.

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CONCLUSIONES

1. Los mutantes *Ljgln2-2* de *Lotus japonicus,* deficientes en glutamina sintetasa plastídica (GS2), experimentan un gran incremento en los niveles de NH_4^+ fotorrespiratorio cuando son transferidos de alto CO_2 a aire, seguido, a los tres días, de un descenso de los mismos, demostrando que la GS2 es una enzima clave para el proceso de reasimilación de amonio fotorrespiratorio y que las plantas mutantes ponen en marcha mecanismos de destoxificación para disminuir los altos niveles de NH_4^+ fotorrespiratorio.

2. En dichas condiciones se produce en los mutantes una represión coordinada de los genes de la fotorrespiración que tiene lugar, posiblemente, para evitar una mayor acumulación de NH_4^+ fotorrespiratorio.

3. Igualmente se produce una inducción de rutas alternativas para la destoxificación de los altos niveles de NH_4^+. Hay una inducción de glutamina sintetasa citosólica, glutamato deshidrogenasa y asparragina sintetasa que pueden ser responsables de la disminución de los niveles de NH_4^+ después de tres días de transferencia a condiciones de fotorrespiración activa.

4. El estudio transcriptómico y metabolómico de la transferencia a condiciones de fotorrespiración activa de plantas silvestres y de los mutantes deficientes en GS2 demuestra que el ciclo fotorrespiratorio, además de su obligada conexión con la fotosíntesis, guarda también, en hojas de *Lotus japonicus,* una importante relación con otros procesos metabólicos incluyendo el metabolismo central del carbono, el metabolismo de aminoácidos y el metabolismo secundario.

5. El estudio comparativo de las transcriptómicas de hojas de plantas de *Lotus japonicus* cultivadas en diferentes fuentes nitrogenadas minerales (NO_3^-, NH_4^+ y NH_4NO_3) tomando como referencia plantas noduladas, muestra que hay un gran número de genes expresados diferencialmente en las distintas nutriciones nitrogenadas, viéndose afectados especialmente genes del metabolismo del nitrógeno, del metabolismo del carbono y del metabolismo secundario. Muchos de estos genes están modulados de forma singular en alguna de las fuentes nitrogenadas utilizadas, lo que pone de manifiesto que cada fuente nitrogenada produce una respuesta transcripcional específica en la planta.

6. La comparación de las transcriptómicas de hojas de plantas silvestres y mutantes en diferentes fuentes nitrogenadas minerales y condiciones de fotorrespiración activa y suprimida muestra que gran parte de los genes que responden a la disminución de la concentración de CO_2 atmosférico también lo hacen con una magnitud similar como consecuencia de la deficiencia en GS2, lo que indica un papel crucial de la esta enzima en el balance C/N de las plantas de *Lotus japonicus*.

7. El estudio transcriptómico del efecto de la sequía en plantas silvestres y mutantes de *Lotus japonicus* demuestra que la respuesta a condiciones de sequía está relacionada con varios aspectos del metabolismo celular, incluyendo la modulación de genes de la fotosíntesis, del metabolismo de aminoácidos, del metabolismo de la pared celular, del estrés oxidativo y del metabolismo del carbono. Por otra parte, los cambios transcriptómicos detectados en los mutantes deficientes en GS2 en la respuesta a sequía son en muchos casos similares a los producidos por los cambios en la actividad fotorrespiratoria de las plantas. Todos estos datos indican una clara interconexión entre el metabolismo del nitrógeno, la fotorrespiración y el estrés hídrico, resaltando la implicación de la GS2 en los cambios transcriptómicos observados.

8. Los estudios transcriptómicos y el uso de redes de co-expresión han permitido identificar varios factores de transcripción que podrían tener papeles clave en la respuesta a la presencia de diferentes fuentes nitrogenadas (factores de transcripción bHLH029, TGA4, dos nodulinas de la familia de nodulinas 21, WRKY50 y dos factores WRKY70), a la disminución de la concentración de CO_2 atmosférico y a la deficiencia de GS2 (factores de transcripción bHLH020 y un dedo de zinc) o a la sequía (factores de transcripción NAC47, ABR1, WRKY40, LBD37 y LBD38, entre otros) en *Lotus japonicus*.

1. The *Ljgln2-2* mutant from *Lotus japonicus*, deficient in plastidic glutamine synthetase (GS2), shows an important increase in the levels of photorespiratoy NH_4^+ when is transferred from high CO_2 to air conditions, followed by a subsequent decrease of NH_4^+ levels after three days, demonstrating that the GS2 is a key enzyme for the reassimilation of photorespiratory ammonium and that the mutant sets up mechanisms of detoxification to diminish the high levels of photorespiratory NH_4^+ accumulated.

2. In the above mentioned conditions, there is a coordinate repression of photorespiratory genes in the mutant, which is produced possibly in order to avoid a further accumulation of photorespiratory NH_4^+.

3. There is also an induction of alternative routes for the detoxification of the high levels of NH_4^+ produced. The induction of cytosolic glutamine synthetase, glutamate dehydrogenase and asparagine synthetase could be responsible for the decrease of the levels of NH_4^+ after three days of transfer to photorespiratory active conditions.

4. Transcriptomic and metabolomic studies of the transfer to photorespiratory active conditions of WT and mutant plants show that the photorespiratory cycle, besides its obliged intertwining with photosynthesis, is linked to several other cellular metabolic processes, including central carbon metabolism, amino acid metabolism and secondary metabolism in *Lotus japonicus* leaves.

5. The comparative transcriptomic analysis of leaves of *Lotus japonicus* plants grown under different mineral nitrogen sources (NO_3^-, NH_4^+ y NH_4NO_3), using nodulated plants as a reference, shows that there are lots of genes that are differentially expressed by the different nitrogen sources including genes involved in nitrogen, carbon and secondary metabolism. Some of these genes are modulated in a singular way on each different nitrogen nutrition, which reveals that each nitrogen source produces a specific transcriptional response in the plant.

6. The comparison of transcriptomic data from leaves of WT and mutants grown under different nitrogen sources, and in photorespiratory active and suppressed conditions, shows that lots of genes are both modulated by the diminishment of CO_2 concentration and by the lack of plastidic GS, thus

indicating that GS2 must have a key role in the C/N balance in *Lotus japonicus* plants.

7. Drought transcriptomics studies of WT and mutant plants show that the response to drought conditions is interconnected with several aspects of cellular metabolism, including modulation of genes involved in photosynthesis, amino acid metabolism, cell wall metabolism, oxidative stress and carbon metabolism. In addition, it is also shown that the transcriptomic changes produced by drought in GS2-deficient mutant plants were similar to the transcriptomic changes produced by the transfer of these mutants to photorespiratory active conditions. All these data indicate a clear interconnection between nitrogen metabolism, photorespiration and drought, with an outstanding implication of GS2 in the transcriptomic changes observed.

8. Transcriptomic studies and the use of co-expression networks allow to identify several transcription factors that could have key roles either in the responses to the different nitrogen nutritions (transcription factors bHLH029, TGA4, two nodulins of the family nodulin21, WRKY50 and two WRKY70), to the diminishment of atmospheric CO_2 concentration and lack of GS2 (transcription factor bHLH020 and a zinc finger) or to drought stress (transcription factors NAC47, ABR1, WRKY40, LBD37 and LBD38, among others) in *Lotus japonicus*.